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Análise genética e dos fatores de virulência de isolados clínicos de *Candida albicans* de pacientes com periodontite crônica portadores de diabetes mellitus tipo II.

Tese apresentada à Faculdade de Odontologia de Piracicaba, Universidade Estadual de Campinas, para obtenção do Título de Doutor em Biologia Buco-Dental, Área de Concentração: Microbiologia e Imunologia.

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“Na vida, não vale tanto o que temos
Nem tanto importa o que somos.
Vale o que realizamos com aquilo
que possuímos e, acima de tudo,
importa o que fazemos de nós.”

(Chico Xavier)

RESUMO

Candida spp. são leveduras comensais que habitam diferentes sítios da cavidade bucal. Em indivíduos saudáveis, sem alterações imunológicas, esses microrganismos não causam doença. Entretanto, diante de condições imunossupressoras, essas leveduras podem se tornar mais virulentas e expressar patogenicidade. Espécies de *Candida* apresentam diversos fatores de virulência, incluindo mecanismos de adesão e invasão celular associado à produção de enzimas que auxiliam na degradação tecidual e facilitam sua proliferação na mucosa bucal. Estudos têm demonstrado a presença de *Candida* sp. em sítios periodontais de pacientes com periodontite crônica, principalmente quando estes são imunologicamente comprometidos. Entretanto, ainda é desconhecido o papel desses microrganismos na patogênese da doença periodontal. Os objetivos do presente trabalho foram: 1) identificar a presença de espécies de *Candida* e periodontopatógenos por PCR em sítios bucais de pacientes diabéticos ou não com periodontite crônica; 2) isolar cepas de *Candida albicans* desses pacientes e avaliá-las quanto à atividade das enzimas proteinase, fosfolipase e hemolisina e os graus de hidrofobicidade da superfície celular, sob diferentes condições atmosféricas, além de realizar a análise genotípica desses isolados; 3) avaliar a capacidade de adesão e invasão de cepas de *Candida albicans* com diferentes graus de hidrofobicidade, em fibroblastos gengivais humanos. As reações de PCR mostraram que os diabéticos tiveram maior prevalência de *Candida* spp. principalmente *C. albicans* e *C. dubliniensis,* e menor freqüência de *Tannerella forsythia,* quando comparado aos pacientes não diabéticos, para bolsa periodontal e furcas. *C. glabrata* e *C. tropicalis* não foram encontradas em sítios periodontais de pacientes não diabéticos. Dos pacientes diabéticos, foram isoladas 128 cepas de *C. albicans*, das quais 51.6% foram determinadas como genótipo B e 48.4% como genótipo A. As condições ambientais consideradas neste estudo, níveis reduzidos de oxigênio ou anaerobiose, não modificaram o tipo de hemólise realizado pelo microrganismo, sendo que a maioria das cepas foi alfa–hemolítica. Nesses ensaios, 100% das cepas em anaerobiose apresentaram as colônias rugosas, enquanto que em ambiente de oxigênio reduzido, houve variação em relação à morfologia e a maioria delas apresentou colônia lisa. Com relação à atividade de proteinase e fosfolipase, cepas de *C. albicans* não

produziram as enzimas na ausência total de oxigênio. Em ambiente com nível reduzido de oxigênio, a maioria das cepas de *C. albicans* foram fortemente produtoras de proteinase e a maioria das cepas foi positiva para fosfolipase. A hidrofobicidade foi mais alta na condição de anaerobiose. A partir desses resultados, foram selecionadas 16 cepas com alta ou baixa hidrofobicidade e avaliadas quanto à capacidade de adesão e invasão em fibroblastos gengivais humanos. Foi verificado que ambos os processos foram maiores nas cepas com alta hidrofobicidade. A produção de óxido nítrico foi maior para as cepas mais hidrofóbicas. Os resultados demonstraram que as espécies de *Candida* podem ser encontradas, em grande proporção, em bolsas periodontais e furcas de pacientes portadores de periodontite crônica, principalmente naqueles acometidos por diabetes *mellitus*. A maioria das cepas de *C. albicans* apresentou atividade enzimática, que atuaria diretamente na degradação tecidual. Além disso, a hidrofobicidade das cepas de *C. albicans* mostrou estar relacionada à maior capacidade de adesão e invasão em fibroblastos. Todos esses fatores de virulência aumentam a patogenicidade da *Candida*, que poderia colaborar na progressão da doença periodontal, principalmente em pacientes imunodeficientes.

Palavra Chave: *Candida* spp., fatores de virulência, periodontite crônica, diabetes mellitus

ABSTRACT

Candida spp. are commensal yeasts that inhabit different sites of the oral cavity. In healthy subjects, without immunological alterations, these microorganisms do not cause disease. However, in immunosuppressive conditions, these yeasts can become more virulent and express pathogenicity. *Candida* species have different virulence factors, including mechanisms of cell adhesion and invasion associated with the production of enzymes that facilitate tissue degradation and their proliferation in oral mucosa. Studies have shown the presence of *Candida* spp. in periodontal sites of patients with chronic periodontitis, especially when they are immunologically compromised. However is still unknown their role in the pathogenesis of periodontal disease. The objectives of this study were: 1) to identify the presence of *Candida* species and putative periodontopathogens by PCR in periodontal sites of diabetic or non-diabetic patients with chronic periodontitis; 2) to isolate strains of *Candida albicans* in these patients and evaluate the proteinase, phospholipase and haemolysin activities and degrees of cell surface hydrophobicity under different atmospheric conditions, besides to performe the genotypic analysis of these isolates; 3) to evaluate the ability of adhesion and invasion of *Candida albicans* strains with different degrees of hydrophobicity, in human gingival fibroblasts. The PCR reactions revealed that diabetics had higher prevalence of *Candida* spp., mainly *C. albicans and C. dubliniensis*, and lower *T. forsythia* frequency, when compared to non-diabetic patients, for both periodontal sites. *C. glabrata* and *C. tropicalis* were not found in periodontal pockets and furcation sites of non-diabetic patients. From diabetic patients, it was isolated 128 strains of *C. albicans* and 51.6% were determined as genotype B and 48.4% as genotype A. The atmospheric conditions, reduced oxygen and anaerobiosis, did not change the type of hemolysis, and the most of strains were alpha-hemolytic. From these assays, 100% of the strains under anaerobiosis showed rough colonies, whereas in an environment with reduced oxyen was no change in relation to morphology and most of them had smooth colony. Considering proteinase and phospholipase activities, *C. albicans* strains did not produce the enzymes in the total absence of oxygen. In reduced oxygen, the majority of *C. albicans* strains were strong proteinase producers and most strains were positive for phospholipase.

Hydrophobicity was higher in anaerobic condition. From these results, 16 hydrophobic or hydrophilic strains were selected and evaluated their ability of adhesion and invasion in human gingival fibroblasts. Both processes were greater in strains with high hydrophobicity. The production of nitric oxide was higher for hydrophobic strains. The results showed that *Candida* species can be found in large proportion, in periodontal pockets and furcation of patients with chronic periodontitis, especially diabetics. The most of *C. albicans* strains showed enzymatic activity, which could act directly on tissue degradation. Moreover, the hydrophobicity of *C. albicans* seems to be related to higher capacity of adhesion and invasion in fibroblasts. All these virulence factors enhance the pathogenicity of *Candida* that could collaborate for the progression of periodontal disease.

Key words: *Candida* spp., virulence factors, chronic periodontitis, diabetes mellitus.

SUMÁRIO

1. INTRODUÇÃO GERAL

As leveduras do gênero *Candida* habitam diversos ecossistemas e estão presentes nos tratos geniturinário e gastrintestinal, unha, pele, brônquios e na cavidade bucal onde podem se estabelecer como microbiota comensal normal sem causar danos ao hospedeiro (Kleinegger *et al*., 1996; Höfling & Rosa, 1999). Porém, doenças sistêmicas, como diabetes melito e AIDS, condições fisiológicas como gravidez, infância ou idade avançada, fatores nutricionais, tratamento com antibióticos de amplo espectro, drogas imunossupressoras e corticosteróides, além de fatores locais como xerostomia e uso de aparelhos protéticos são condições que predispõem ao desenvolvimento de infecções por *Candida* spp. (Tekeli *et al*., 2004; Manfredi *et al*., 2006).

Uma grande parte da população adulta saudável abriga levedura do gênero *Candida* na cavidade bucal. A mucosa é considerada o reservatório principal, porém estudos têm mostrado que espécies de *Candida* podem estar co-agregadas a bactérias em biofilme dental podendo ser um fator importante para manifestações de candidoses e para o processo de colonização tanto de cavidades de cáries quanto de bolsas periodontais (Höfling *et al*., 2004). Segundo alguns autores, a presença de leveduras em regiões subgengivais pode contribuir para a patogênese da doença periodontal ou então aumentar a chance de candidemia, principalmente em casos de depressão do sistema imune (Hannula *et al*., 2001; Reynaud *et al*., 2001).

A periodontite é uma doença inflamatória infecciosa caracterizada pela destruição irreversível dos tecidos de suporte dos dentes (epitélio juncional, tecido conjuntivo gengival, ligamento periodontal e osso alveolar). A periodontite não tratada, eventualmente, pode levar à perda do dente e está associado a uma microbiota subgengival bastante diversificada e complexa envolvendo bactérias Gram-positivas, Gram negativas além de anaeróbios facultativos e possivelmente leveduras. Pelo menos cerca de 500 espécies de microrganismos foram isolados a partir do biofilme subgengival (Haffajee & Socransky, 1994; Kornman *et al.,* 1997). Acredita-se que a maioria desses patógenos são comensais e apenas um pequeno número poderia ser potencialmente patogênico e oportunista. A periodontite crônica resulta de uma complexa interação entre os microrganismos

causadores da infecção e a resposta do hospedeiro, ocorrendo assim liberação de mediadores inflamatórios que resulta na destruição do tecido periodontal (Kornman *et al*., 1997; Chen *et al.*, 2000; Järvensivu *et al.*, 2004).

Embora *Candida* spp. possam ter um papel como patógeno periodontal, esse microrganismo não tem recebido o enfoque necessário, mesmo tendo reconhecida capacidade de adesão ao epitélio, invasão do tecido conjuntivo gengival, produção de fatores de virulência e indução de reações inflamatórias (Reynaud *et al*., 2001 ; Jarvensivu *et al*., 2004). Jarvensivu *et al*., (2004) estudaram, através de imunohistoquímica, a ocorrência e a extensão da penetração de *Candida albicans* nos tecidos periodontais de pacientes com periodontite crônica, em tecidos gengivais, coletados durante cirurgias periodontais a retalho e observaram a presença de hifas penetrando no tecido conjuntivo periodontal. Os autores sugerem que alterações ambientais podem favorecer a germinação de hifas que possuem maior capacidade de aderir e penetrar nos tecidos do hospedeiro e que a bolsa periodontal e o fluido crevicular formam um ambiente favorável para a germinação dessas estruturas morfológicas. Ainda para esses autores, a *C. albicans* poderia ter um papel na infra-estrutura do biofilme subgengival, e em sua adesão aos tecidos periodontais, visto que são mais resistentes aos mecanismos imunes do que os demais microrganismos.

Tem sido descrito na literatura que a proporção de leveduras em bolsas periodontais é similar a de algumas bactérias periodontopatogênicas, sugerindo, assim, o possível papel de *Candida* spp. na patogênese da doença (Dahlen & Wikström, 1995). Em relação à proporção das diferentes espécies de *Candida*, *C. albicans* é a levedura encontrada mais comumente nas populações humanas, e essa espécie tem sido considerada um agente infeccioso importante, principalmente pela capacidade de sobreviver, disseminada geograficamente, em estado de comensalismo, ou mesmo causando infecções em vários grupos populacionais humanos e em diferentes sítios anatômicos, cada qual com suas condições ambientais distintas. Ainda, a versatilidade desse microrganismo faz com que o espectro de doenças causadas por *C. albicans* seja maior do que o de outras espécies microbianas oportunistas, capazes de colonizar apenas um ou poucos sítios do hospedeiro (Calderone & Fonzi, 2001). *C. albicans* tem sido frequentemente isolada da cavidade bucal

de pacientes com diabetes mellitus. Manfredi *et al.*, (2002) isolaram *C. albicans* de 77 % dos pacientes insulino-dependentes. Um número relevante de fatores incluindo deficiência no sistema imune tem sido associado com a presença da levedura em pacientes diabéticos como o tipo, a duração e o grau de controle glicêmico (Manfredi *et al*., 2002).

Diabetes mellitus é uma doença metabólica caracterizada por níveis elevados de glicose no sangue; que ocorre por causa da deficiência de insulina secretada ou por aumento da resistência a insulina (Lamster *et al*., 2008). Altas taxas de glicose nos tecidos moles, além de proporcionar baixa resistência desses tecidos ao estresse, podem resultar em necrose da gengiva marginal como resultado da circulação sanguínea deficiente. Evidências sugerem existir correlação positiva entre diabetes mellitus e destruição periodontal principalmente em diabéticos não controlados (Bennatti *et al*., 2003). Diabetes mellitus pode prejudicar a função dos leucócitos polimorfonucleares que podem predispor o paciente diabético a um risco maior de infecções, incluindo a doença periodontal e infecções orais por *Candida* spp. (Urzúa *et.al*. 2008).

Vários fatores contribuem para a patogenicidade de leveduras do gênero *Candida*, entre eles é possível citar a capacidade de adesão a células epiteliais e endoteliais, a germinação, a produção de proteinases e fosfolipases, hemolisinas, hidrofobicidade, co-agregação com outros microrganismos, entre outras (Ghannoum, 2000; Sugita *et al*., 2002). Ainda segundo Ghannoum (2000), o balanço entre fatores de virulência e condições ligadas ao hospedeiro pode determinar a tendência de espécies de *Candida* em se tornar saprófitas ou patogênicas. A adesão de microrganismos às superfícies mucosas do hospedeiro é um pré-requisito para a colonização e infecção (Samaranayake & Mac Farlane, 1982). Esse mecanismo permite que o microrganismo evite o deslocamento pela ação mecânica da saliva (Ellepola *et al*., 1999). A aderência da levedura à superfície da mucosa do hospedeiro ocorre provavelmente pela interação entre adesinas do microrganismo e receptores das células epiteliais (Olsen, 1990).

A produção de enzimas, como proteinases e fosfolipases, representa outro mecanismo de patogenicidade de *C. albicans*, pois estas facilitam a invasão da levedura em tecidos. As proteinases constituem uma família de enzimas capazes de degradar vários substratos fisiologicamente importantes tais como albuminas e imunoglobulinas (Cassone

et al., 1987). Shimizu *et al*., (1996) observaram que cepas de *C. albicans* que não produziam essa enzima eram menos virulentas. As fosfolipases são enzimas hidrolíticas que degradam fosfolipídeos, e por esses compostos serem constituintes das membranas celulares, a liberação dessas enzimas pode levar à lise celular (Samaranayake *et al*., 1984). Estudos demonstraram que a produção de fosfolipases pode estar relacionada à capacidade de adesão em *C. albicans* (Samaranayake *et al*., 1984; Barret-Bee *et al*.; 1985), entretanto, tal associação ainda permanece controversa (Barros *et al*., 2008).

Devido ao relevante papel que as espécies de *Candida* têm desempenhado na ecologia da cavidade bucal, e a importância de estudos sobre a distribuição desses microrganismos nas populações e nos diferentes sítios anatômicos, a presente tese teve como objetivos: 1) identificar a presença de espécies de *Candida* e periodontopatógenos por PCR em sítios bucais de pacientes diabéticos ou não com periodontite crônica; 2) isolar cepas de *Candida albicans* desses pacientes e avaliá-las quanto à atividade das enzimas proteinase, fosfolipase e hemolisina e os graus de hidrofobicidade da superfície celular, sob diferentes condições atmosféricas, além de realizar a análise genotípica desses isolados; 3) avaliar a capacidade de adesão e invasão de cepas de *Candida albicans* com diferentes graus de hidrofobicidade, em fibroblastos gengivais humanos.

2. CAPÍTULOS

Esta tese está baseada na Deliberação CCPG/001/98/Unicamp e na aprovação pela Congregação da Faculdade de Odontologia de Piracicaba em sua 105ª Reunião Ordinária em 17/12/2003, que regulamenta o formato alternativo para tese de Doutorado e permite a inserção de artigos científicos de autoria do candidato.

Assim sendo, esta tese é composta de 4 capítulos contendo artigos científicos aceitos ou em fase de submissão para revistas internacionais, conforme descrito a seguir:

Capítulo 1

Artigo "***Candida* ssp. in periodontal disease – a brief review"**

Este artigo foi aceito para publicação pelo periódico *Journal of Oral Science* em abril/2010.

Capítulo 2

Artigo **"Prevalence of putative periodontopathogens and *Candida* spp. in diabetic and non-diabetic patients with chronic periodontitis."**

Capítulo 3

Artigo **"Genetic and phenotypic evaluation of *Candida albicans* strains isolated from subgingival biofilm of diabetic patients with chronic periodontitis".**

Capítulo 4

Artigo "**In vitro evaluation of adhesion and invasion of *Candida albicans* in gingival human fibroblasts."**

3. CAPÍTULO 1

Candida spp. in periodontal disease: a brief review

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Abstract

Although the main reservoir of *Candida* spp. is believed to be the buccal mucosa, these microorganisms can coaggregate with bacteria in subgingival biofilm and adhere to epithelial cells. Such interactions are associated with the capacity of *Candida* spp. to invade gingival conjunctive tissue, and may be important in the microbial colonization that contributes to progression of oral alterations caused by diabetes mellitus, some medications, and immunosuppressive diseases such as AIDS. In addition, immune deficiency can result in proliferation of *Candida* spp. and germination of forms that are more virulent and have a higher capacity to adhere to and penetrate cells in host tissues. The virulence factors of *Candida* spp. increase host susceptibility to proliferation of these microorganisms and are likely to be important in the study of periodontal disease. Herein, we briefly review the literature pertaining to the role of *Candida* spp. in periodontal disease, and consider the main virulence factors, the host immune response to these microorganisms, and the effect of concomitant immunosuppressive conditions.

Keywords: *Candida* spp., periodontal disease, immune system, virulence factors

1. Introduction

The *Candida* spp. are opportunistic pathogens that can cause disease in hosts who are compromised by underlying local or systemic pathological processes (1,2). *Candida albicans* is the species most often associated with oral lesions, but other *Candida* spp., including *C. glabrata, C. tropicalis, C. parapsilosis, C. krusei,* and *C. dubliniensis* have also been isolated (2) in the saliva of subjects with and without oral candidiasis. The isolation of *Candida* from the oral cavity does not imply the presence of disease (3). Fungal organisms commonly colonize the tongue, palate, and buccal mucosa. Such colonization may also occur in subgingival plaque of adults with periodontitis (4).

The *Candida* spp. have virulence factors that facilitate colonization and proliferation in the oral mucosa and, possibly, in periodontal pockets. These fungal organisms can coaggregate with bacteria in dental biofilm and adhere to epithelial cells. These interactions, which are associated with their capacity to invade gingival conjunctive tissue, may be important in microbial colonization that contributes to progression of oral diseases (5,6). In addition to these properties, *Candida* spp. also produces enzymes, such as the collagenases and proteinases that degrade extracellular matrix proteins, and immunoglobulins (5). Barros et al. (2008) (7) investigated the genetic diversity and production of exoenzymes in *C. albicans* and *C. dubliniensis* isolated from the oral cavity of systemically healthy patients with periodontitis. They verified that genetically homogeneous strains of *C. albicans* were present in the oral cavity of these patients and that these strains were capable of producing high levels of exoenzymes.

The attachment of *Candida* spp. to oral epithelium is the first step in the colonization process, after which local and systemic host defense mechanisms are activated to combat fungal proliferation and infection. This local defense comprises lactoferrin, β-defensins, histatins, lysozyme, transferrin, lactoperoxidase, mucins, and secretory immunoglobulin A (sIgA) (8). The innate immunity system also recognizes specific cell-wall surface proteins of *Candida* spp. and participates in the response against *Candida* infection (9).

Species of *Candida*, especially *C. albicans*, have been recovered from periodontal pockets in 7.1% to 19.6% of patients with chronic periodontitis (4,10). Urzúa et al. (11)

observed that *C. albicans* and *C. dubliniensis* were capable of colonizing periodontal pockets in patients with chronic periodontitis, while only *C. albicans* was identified in the subgingival microflora of healthy individuals and patients with aggressive periodontitis. Cancer, diabetes mellitus, and immunosuppressive conditions such as acquired immunodeficiency syndrome (AIDS) increase host susceptibility to these infections. Feller et al. (12) observed higher prevalences of *Candida* spp. in the oral cavity, and specifically in the subgingival biofilm, of human immunodeficiency virus (HIV)-seropositive patients. In the present report, we briefly review the literature on the role of *Candida* spp. in periodontal disease, consider the main virulence factors and host immune responses to these microorganisms, and describe the effects of concomitant immunosuppressive conditions.

2. Virulence factors of *Candida* spp.

Candida albicans is frequently found in humans, and often resides on skin, mucosa, and normal gingival sulcus of otherwise healthy individuals. In compromised hosts, however, *Candida albicans* can cause serious disease, ranging from deep-seated mucosal infection to systemic infections (1,13). Several factors have been proposed as virulence factors of *Candida* spp., including adhesion; phenotypic diversity; hyphal formation; production of phospholipases, proteinases, or other metabolites; synergistic coaggregation or competition with bacteria; and mechanisms for adaptation in the host environment (13). The capacity of *Candida* spp. to adhere to different cells is important in its dissemination, infection, and persistence in oral and other tissues. Nikawa et al. (14) quantitatively evaluated the adhesion of oral isolates of *C. albicans*, *C. tropicalis*, and *C. glabrata* to human gingival epithelial cells, gingival fibroblasts, and pulmonary fibroblasts. They observed that most *Candida* strains had significantly higher adherence to gingival epithelial cells than to either type of fibroblast. However, environmental factors such as diet, composition of body fluids, and presence of antifungal agents may also cause changes in the cell surface and thereby modulate *Candida* adhesion.

Candida spp. have developed several virulence traits that facilitate invasion of host tissues and evasion of host defense mechanisms. One such group of virulence factors is the

hydrolytic enzymes, which are secreted extracellularly by these microorganisms. Important hydrolytic enzymes include the phospholipases and secreted aspartyl proteinases (SAPs): 7 phospholipase genes (PLA, PLB1, PLB2, PLC1, PLC2, PLC3, and PLD1) and 10 SAP genes (SAP1 to SAP10) have been identified in *C. albicans* (15,16). Although their roles in pathogenesis have not been fully elucidated, it is known that phospholipases facilitate adherence to tissue, in addition to degrading phospholipids present in the cell membrane, which ultimately leads to cell lysis. Similarly, SAP9 and SAP10 collaborate in adherence, tissue damage, and evasion of host immune response (17). Adherence to epithelial cells is the first step in *C. albicans* colonization, and is followed by the establishment of mucocutaneous infection. It has been proposed that SAPs produced by *C. albicans* digest the surface of epithelial cells, thereby providing an entrance into the cell (18). In an investigation of the interaction between this microorganism and epithelial cells, it was found that hyphae are the invasive form of the organism, and that blastospores are generally found either on the surface of or between epithelial cells (18). Invasion of epithelial cells by *C. albicans* can also occur by means of endocytosis, in which pseudopods surround the organism and pull it into the cell (19). Although both yeast and hyphal-phase organisms are capable of inducing endocytosis, hyphae are more efficient at stimulating this process, suggesting that, in this form, *C. albicans* expresses specific invasive molecules on its surface, and that these bind to 1 or more epithelial cell receptors and induce endocytosis (20).

In addition to these virulence factors, coaggregation has been observed between some species of *Candida* and other oral microorganisms, but the extent of this coaggregation depends on growth conditions such as temperature (21). These interspecies interactions may be important in the microbial colonization that contributes to the progression of oral diseases. It has been suggested that the initial interspecies association is followed by a tight adhesion-receptor interaction, mediated by a mannoprotein in *C. albicans* (22). Hydrophobic proteins in the polysaccharide matrix of the *C. albicans* cell wall contribute to the strength of this adhesion receptor, and increase the pathogenicity of the yeast (22). Indeed, hydrophobicity has been found to be correlated with increased

virulence in *Candida* spp., because hydrophobic cells are more adherent to host cells and substrates, including mucin and extracellular matrix proteins (23).

Because microorganisms exist in polymicrobial communities, the capacity of yeasts to coexist with commensal or pathogenic bacteria is an important virulence factor. The quantitative and qualitative characteristics of coexisting microorganisms may therefore influence *Candida* biofilm formation. Thein et al. (24) evaluated the effects of oral bacteria, including the periodontopathogens *Prevotella nigrescens* and *Porphyromonas gingivalis*, on the development of *Candida albicans* biofilm *in vitro*. They observed a reduction in yeast counts when these microorganisms were cocultured with *Candida* biofilm, possibly because metabolites produced by anaerobes interfere with biofilm physiology or because the physical presence of bacteria inhibits biofilm growth. In assessing the effectiveness of antifungals, studies using mixed biofilms are of greater value than those using isolated species.

Hemolysin is another virulence factor that contributes to the pathogenesis of *Candida*. The secretion of hemolysin, followed by iron acquisition, facilitates hyphal invasion in disseminated candidiasis (25). An elevated blood glucose concentration may contribute, directly or indirectly, to increased hemolysin activity among *C. albicans* isolates in diabetic patients (26). In addition to physiological factors, the study of genotypic diversity, as assessed by molecular typing techniques, has become fundamental in elucidating the epidemiology of *Candida* isolates. Pizzo et al. (27) suggested that heterogeneity within subgingival *C. albicans* isolates results not only from the spreading of *Candida* microorganisms from saliva or biofilm, but also from new strains adapting to subgingival pockets and developing different virulence properties.

Studies have demonstrated that *Candida* spp. can adapt to an adverse host environment by altering pH, oxygen concentration, and nutrient availability. Environmental pH has profound effects on cells. Firstly, proteins have pH optima for activity, and become nonfunctional when pH is changed (28). Alterations in pH also stress fungi by disturbing the acquisition of nutrients, including iron (28). Iron is stored intracellularly in ferritin complexes. It is bound by transferrin in tissues and by lactoferrin on mucosal surfaces, and is an important facet of innate immunity (28). The effects of pH and innate immunity

limit iron acquisition by pathogenic fungi. However, signaling pathways allow fungi to sense alterations in environmental pH and change the expressions of the genes—that regulate modifications in the morphology of *Candida* spp. (i.e., PHR1 and PHR2), resulting in either acidic pH-promoting yeast cell growth or neutral-alkaline pH-promoting filamentous growth (28). Moreover, *Candida* spp. can grow in both aerobic and anaerobic conditions, and have developed adaptive mechanisms to survive in both conditions. Oxygen can generate reactive products during infection and induce an oxidative stress response. Treatment of *C. albicans* with low concentrations of either hydrogen peroxide or menadione (a superoxide-generating agent) induces a redox potential with the activation of antioxidant enzymes, which protects cells from the lethal effects of a subsequent challenge with higher concentrations of these oxidants (29). The presence of anaerobic environments, such as those in root canal systems and periodontal pockets, can lead to polymicrobial infections. Rosa et al. (30) found that SAP secretion consistently increased in cultures of *C. albicans* strains, when strains recovered from periodontal pockets and intraoral sites not associated with the periodontium were grown under anaerobic conditions. This suggests that oxygen concentration in the atmosphere surrounding cells influences the virulence attributes of *C. albicans.*

The presence of a large amount of carbohydrate in the oral cavity influences several virulence factors of *Candida* spp. Incubation in sucrose, glucose, fructose, or maltose promotes adhesion of *C. albicans*, *C. tropicalis*, and *C. krusei* to epithelial cells (31), increases acid production, and lowers pH, with consequent activation of acid proteinases and extracellular phospholipases—factors involved in yeast adhesion (32). Polysaccharides such as as rhamnose, mannose, and N-acetyl-glucosamine are present in the skeletal cell wall (33) and biofilm matrix (34), which make them targets for the development of therapies capable of disrupting cells and biofilms. In the tridimensional structure of the biofilm, there are a variety of covalently linked cell wall proteins (CWPs) that play a direct role in the response to several stress conditions (1). Genomic transcript analysis and, to a lesser extent, cell wall proteome analysis have shown that the response of *C. albicans* to certain forms of stress often includes dramatic changes in the protein levels of CWPs,

which confirms that these proteins play a crucial role in virulence and that their expression is tightly controlled (1).

3. Recognition of *Candida* spp. by the immune system

The oral epithelium is an effective primary barrier against invasion by a number of oral commensals, including *Candida* spp. Once this barrier is breached, other innate immune mechanisms, such as the interleukins (ILs) and colony-stimulating factors of epithelial cells, come into play (35). Other local defense mechanisms against mucosal infection include the salivary proteins (e.g., lactoferrin, β-defensins, histatins, lysozyme, transferrin, lactoperoxidase, and mucins) and secretory immunoglobulin A (sIgA), which are activated in the immune response against *Candida* infection (8). These salivary proteins can impair adhesion and growth of *Candida* in the oropharyngeal cavity (8). The adherence of *C. albicans* to oral epithelia is the first step in the infection process and enables the yeast to overcome the normal flushing mechanism of body secretions (36). Host defense mechanisms against mucosal candidiasis are not well understood, but include both innate and adaptive responses.

Phagocytic cells recognize pathogens by means of a variety of pattern recognition receptors (PRRs), including toll-like receptors (TLRs). The TLR family is a class of 13 receptors that are abundantly expressed on innate immune cells—such as macrophages, dendritic cells (DCs) (37), monocytes, neutrophils—and in the mucosal epithelium of the mouth, middle ear, and nasopharynx (38). Neutrophils strongly express phagocytic receptors such as complement receptor 3 (CR3) and Fcγ-receptors (FcγRs), which facilitate uptake into the fungus. Complement binding and activation is mediated by the alternative pathway. Complement activation is mainly important for chemotaxis and opsonization in *C. albicans*, but not in *C. albicans* lyses, in which it is prevented by the thick and complex cell wall (8, 39). Several membrane-bound receptors localized in macrophages, monocytes, neutrophils, and dendritic cells contribute to the phagocytosis of *C. albicans*. These include dectin 1, dendritic cell-specific intercellular adhesion molecule-3–grabbing non-integrin (DC-SIGN), and mannose receptor (MR). Dectin 1 is a myeloid-expressed transmembrane receptor that, in response to fungi, induces a respiratory burst resulting in production of

toxic oxidants (40). DC-SIGN is a receptor that is specifically expressed on the cell membrane of dendritic cells (DC), which have been shown to directly mediate uptake of fungal particles in transfected cell systems (40). MR is found in macrophages and dendritic cells, but its ability to mediate phagocytosis has recently been questioned (41). Recognition of *C. albicans* by the immune system triggers the production of the Th1 and Th2 cytokines, mainly by $CD4^+$ T cells. The TLRs (TLR2, TLR4, TLR6, and TLR9) are also involved in triggering these cytokine responses—which are linked to mechanisms of innate and acquired immunity—together with the participation of cells such as macrophages, neutrophils, and dendritic cells (9).

In general, upon recognition of microbial structures, TLRs activate either the NFκB (nuclear factor κB) or MAPK (mitogen-activated protein kinase) pathway, which leads to the production of different cytokines (42). Furthermore, some studies have found that TLR2 and TLR4 play roles in modulating Th1 and Th2 immune responses against *Candida*. There is also evidence indicating that TLR2 can recognize blastoconidia and hyphae of *C. albicans*, while TLR4 recognizes only blastoconidia. Van der Graaf et al. (43) showed that the recognition of hyphae and blastoconidia by TLR2 induces a Th2 immune response, culminating in the synthesis of IL-10 and IL-4 cytokines, which are capable of inhibiting the Th1 pattern response. As a result, elimination of *C. albicans* is slowed and the microorganism can disseminate in the host. Blastoconidia are recognized by TLR4, which markedly stimulates proinflammatory cytokines such as IFN-γ (interferon-γ), IL-6, TNF-α (tumor necrosis factor- α), and IL-12. Th1 or Th2 responses seem to be an important determinant of the host's ability to contain infection. Th1 responses are correlated with protection of the host, and the progression of infection is associated with the predominance of Th2 responses (44). A balance between Th1 and Th2 cytokines may thus be important in ensuring optimal antifungal protection while minimizing immune-mediated damage. Studies have shown that *C. albicans* induces immunosuppression through TLR2-mediated IL-10 release, and that this can lead to the generation of $CD4^+CD25^+$ regulatory T cells with immunosuppressive potential (9). In addition, *in vivo* models indicate that regulatory T cells attenuate Th1 antifungal responses, induce tolerance to the fungus, and participate in the development of long-lasting protective immunity after yeast priming (8).

It must be emphasized that in candidiasis, the different mechanisms of the immune system, as described above, act synergistically, i.e., they cooperate with and modulate each other in the process of combating fungal infection (45). The immune response to *Candida* spp. is related mainly to the different cytokines and chemokines produced by Th1 or Th2 cells; however, concomitant humoral immune responses to oral candidiasis and *C. albicans*-specific IgA and IgG antibodies have been observed (46,47). The exact mechanisms by which these antibodies protect against *Candida* infection are unknown, but are likely to include inhibition or germ tube formation, opsonization, neutralization of virulence-related enzymes, and direct yeast activity (45).

Inhibition of *C. albicans* adhesion to host surfaces is mediated by antibodies, and the extent of this inhibition has been analyzed in saliva samples. Fungi can also activate the complement system by the classical and alternative pathways, with deposition of C3 on the cell fungal surface. Complement activation facilitates the recruitment of phagocytes to infected tissues and enhances their anticandidal activity (8). It has been observed that the protective potential of antibodies with enhanced phagocytosis and the killing of fungus depends upon epitope specificity, serum titer, and the ability of the complement system to bind in the fungal surface (8).

Few studies have been designed to investigate the immune response against *Candida* spp. in periodontal disease. Phagocytosis and killing of *C. albicans* by polymorphonuclear (PMN) cells were compared in patients who received organ transplants and those with periodontal disease. PMN cells were isolated and, after 20 min incubation, the phagocytosis of *C. albicans* and the intracellular killing rate were determined. The authors found no significant decrease in phagocytosis between transplant patients and those with periodontal disease. However, the killing activity of PMN cells was lower in these 2 patient groups than in healthy controls, an effect that was unrelated to the severity of periodontal disease (48). These results suggest that a reduction in killing activity, whether spontaneous or drug-induced, contributes to the development of periodontal disease (42). Using an enzyme-linked immunosorbent assay and whole unstimulated and stimulated saliva, Hägewald et al. (49) analyzed total IgA, IgA subclass 1, IgA subclass 2, and IgA reactivity to *Aggregatibacter actinomycetemcomitans*, *Treponema denticola*, and *C.*

albicans. Significantly low concentrations and secretion rates of total salivary IgA, IgA1, and IgA2 were found in aggressive periodontitis. For all 3 microorganisms tested, the proportion of bacteria-reactive IgA in total IgA was significantly higher in the aggressive periodontitis group. In saliva, the pattern of humoral IgA response to *C. albicans* was similar to that of the *A. actinomycetemcomitans* and *T. denticola* antibodies. In addition, during activation of the bacteria-reactive humoral immune system in saliva, the authors observed inhibition of total secretory IgA, in particular IgA subclass 1, in aggressive periodontitis. Further studies are needed to elucidate the mechanisms of the immune system that control and eliminate *Candida* spp. from the host.

4. Immunosuppressive conditions and proliferation of Candida spp. in periodontal patients

Periodontal alterations are believed to be the result of an exacerbated immune response against host tissues. Changes in cellular and humoral immune responses (50) may allow different species, such as *Candida,* to colonize the subgingival environment (51). It has been reported that the proportion of yeasts in periodontal pockets is similar to that of some bacterial periodontopathogens, which suggests a role for *Candida* spp. in the pathogenesis of the disease (52,53). However, it is not yet possible to determine the role of *Candida* in the development or progression of periodontal disease, because only few studies have investigated the presence of yeasts in periodontitis patients, and they do not clear indicate whether their patients suffered the chronic or aggressive form of the disease (4,3,52). It is unclear if yeasts contribute to the development of periodontal disease, or if they show specificity for the chronic or aggressive forms of the disease (6,10,54,55,56). However, individuals with cancer, diabetes mellitus, and immunocompromising conditions such as HIV/AIDS are more susceptible to a wide spectrum of infections, including fungal infections (12, 57,58).

Periodontal conditions were studied in 2 cross-sectional studies of adult, insulin-dependent, diabetics and age-and sex-matched controls (57). In one study, 154 diabetics and 77 controls participated; the other comprised 83 diabetics and 99 controls. There was a higher percentage of individuals with severe periodontal disease in the diabetic group than

in the control group (57). However, there was no association between diabetes mellitus, periodontal disease, and the presence of *Candida* spp. In addition, a moderate increase in the glucose content of saliva did not result in higher mean numbers of *C. albicans*. Similar results were obtained by Yuan et al. (59), who found no significant differences between diabetic and nondiabetic individuals in the prevalence of a number of microorganisms, including *C. albicans*.

In our previous study (60), using a polymerase chain reaction (PCR) assay, we found that quantities of some *Candida* spp. were higher in chronic periodontal disease patients with diabetes than in those without diabetes. Among diabetic patients, *C. albicans* was found in 57%, *C. dubliniensis* in 75%, *C. tropicalis* in 16%, and *C. glabrata* in 5% of periodontal pockets. Among nondiabetic patients, *C. albicans* and *C. dubliniensis* were present in 20% and 14% of periodontal sites, respectively; there was no evidence of *C. tropicalis* or *C. glabrata* colonization. Urzúa et al. (11) used phenotypic and genotypic methods to analyze the composition of yeast microbiota in the mucosa and subgingival sites of healthy individuals and in patients with aggressive and chronic periodontitis. Although the profiles of the species present in the mucosa of the 3 groups varied, they noted that only *C. albicans* and *C. dubliniensis* were capable of colonizing periodontal pockets in patients with chronic periodontitis, and that only *C. albicans* was identified in the subgingival pockets of healthy individuals and patients with aggressive periodontitis. It has been reported that the proportion of yeasts in periodontal pockets is similar to that of some bacterial periodontal pathogens, which suggests a role for *Candida* spp. in the pathogenesis of this disease (11).

Certain *Candida* spp. are believed to be commensal organisms within the oral cavity. Indeed, the prevalence of oral yeast in the general population is about 34%. However, certain patient subgroups have higher levels of oral colonization. Peterson et al. (61) noted that the prevalence of oral yeast in hospitalized patients was 55%. Oral yeast carriage is particularly common in patients with advanced cancer, among whom reported levels of oral colonization range between 47% and 87%.

In 24 patients with acute periodontal infection and chemotherapy-induced myelosuppression, high concentrations of microorganisms were detected in subgingival

pockets. *Staphylococcus epidermidis, C. albicans, S. aureus,* and *Pseudomonas aeruginosa* were predominant, and combinations of these were detected in some patients (62). Drugs such as corticosteroids, azathioprine, and cyclosporine are used to prevent the rejection of transplanted organs; however, these agents can alter the immune system and modify the characteristics of dental biofilm, thereby altering its effects on periodontal tissues. Renal transplant patients who received immunosuppressive drugs had more periodontal inflammation than did immunocompetent subjects (62). Infections can also occur in immunosuppressed patients, and these frequently involve microorganisms that have little or no pathologic significance in immunocompetent hosts. The prevalence of microorganisms in the periodontal sites of patients receiving immunosuppressive therapy may increase in local disease or disseminated infections, and some cultivable species, including *A. actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Campylobacter rectus, Fusobacterium* spp., *Streptococcus* spp., *Pseudomonas* spp., and *Candida* spp., have been detected (62).

The *Candida* spp. are one of the most common AIDS-defining fungal opportunistic infections in HIV-positive individuals. One study found that the prevalence of *Candida* spp. in subgingival sites was 42.3% in HIV-positive children and 7.1% in control individuals (63). In another study, the authors observed a higher prevalence of *Candida* species in the oral cavity of HIV-seropositive patients, specifically in the subgingival biofilm, although the prevalence of periodontal disease in HIV-seropositive and HIV-seronegative subjects was very similar (12). Using conventional mycological methods and a specific PCR assay, Jewtuchowicz et al. (64) studied immunocompromised patients, such as those with advanced HIV infection, to identify the different species of yeast present at periodontal disease sites. Among the 76 fungal organisms isolated, 10.5% were *C. dubliniensis*, which was present in 4.4% of patients studied; *C. albicans* was the most frequently isolated yeast species.

The *Candida* spp. are ubiquitous fungal organisms that often colonize the oral mucosa of normal individuals, without causing disease. These opportunistic microorganisms might influence the inflammatory process, as they possess several virulence factors by which they invade tissues and evade host defense mechanisms, thereby

facilitating proliferation and release of exoenzymes that promote tissue degradation. Moreover, in immunosuppressed patients, the higher prevalence of *Candida* spp. (mainly *C. albicans*) in the oral cavity, and specifically the subgingival biofilm of periodontal pockets, could indicate their coparticipation in the progression of periodontal disease in these patients.

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4. CAPÍTULO 2

Prevalence of putative periodontopathogens and *Candida* spp. in diabetic and non-diabetic patients with chronic periodontitis

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Abstract

Alterations that cause deficiency on the immune system, such as diabetes mellitus, can induce the proliferation of *Candida* spp. and germination of more virulent forms with higher capacity to adhere and penetrate in the host tissues cells. In the oral cavity, these microorganisms can co-aggregate to bacteria in dental biofilm and to contribute for microbial colonization process of periodontal pockets. Studies have showed positive correlation between diabetes mellitus and destruction of the periodontal tissue, mainly in non-controlled diabetic patients. The aims of this study were to identify by PCR the presence of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* and *Tannerella forsythia*, and four different species of *Candida* (*C. albicans, C. dubliniensis, C. glabrata* and *C. tropicalis)* in periodontal pockets and furcation sites of type 2 diabetics and non-diabetics patients with generalized chronic periodontitis. Samples of subgingival biofilm were obtained from periodontal pockets and furcation sites of 10 diabetic and 10 non-diabetic patients with chronic periodontitis and submitted to phenol-chloroform DNA extraction and PCR analysis using specific primers. The results revealed that diabetics had higher prevalence of *Candida* spp., mainly *C. albicans and C. dubliniensis,* and lower *T. forsythia* frequency, when compared to non-diabetic patients, for both periodontal sites ($p<0.05$, Chi-square test). *C. glabrata* and *C. tropicalis* were not found in periodontal pockets and furcation sites of non-diabetic patients. The results demonstrated a strong colonization of *Candida* spp. in periodontal sites of diabetic patients with generalized chronic periodontitis with a higher prevalence of *C. dubliniensis* followed by *C. albicans*.

Keywords: periodontal disease, diabetes mellitus, *Candida* spp., periodontopathogens, PCR

1. Introduction

Periodontitis is an infectious disease caused by the interaction between microorganisms, their products and the exacerbated host immune response, resulting in irreversible destruction of the tooth-supporting tissues. [1] This process is associated with a widely diverse and complex subgingival microbiota including both Gram-positive and Gram-negative bacteria, facultative or anaerobic organisms. In particular, bacteria *Tannerella forsythia* (*Tannerella forsythensis* or formerly *Bacteroides forsythus*), *Porphyromonas gingivalis* and *Treponema denticola*, called red complex,[2] have been assigned important role in various forms of periodontal disease in adults.[2,3] Changes in cellular and humoral immune responses may allow different species, such as yeasts and virus, to colonize the subgingival environment.[4,5] In the oral cavity, yeasts commonly colonize the tongue, palate and buccal mucosa and some studies have also found it in subgingival plaque of adults with periodontitis.[6,7,8,9,10,11] Using scanning electron microscopy, yeasts were detected in 26 of 60 periodontal tissue samples from 12 patients with periodontitis juvenile.[12] Reynaud et al. (2001) [13]verified that the prevalence of subjects with yeasts in periodontal pockets was 15.6%. It has been reported that the proportion of yeasts in periodontal pockets is similar to that of some bacterial periodontopathogens.[5,8,9,14]

Candida spp. is an opportunistic pathogen that causes disease in hosts who are compromised by underlying local or systemic pathological processes.[15] Yeasts infections are linked to a number of local and general predisponing factors as diabetes mellitus, HIV, renal transplant or cytotoxic treatment.[16,17,18,19] Diabetes mellitus is a metabolic disease characterized by hyperglycemia due to defects in insulin production, insulin action, or both. The development of diabetes can impair the function of polymorphonuclear leukocytes predisponding patients to greater risk to develop opportunist diseases.[20] Numerous oral complications, such as decreased function of salivary glands (xerostomia), burning sensation and periodontal disease, have been related to diabetes mellitus and yeast colonization.[21] Species of *Candida* have been frequently isolated from the oral cavities of diabetics and the highest rate of colonization occurs in patients with poor glycemic control.[22,23] Besides, *Candida* spp. is markedly increased in mucosa and saliva of diabetics

in comparison with non-diabetic patients.[20,24] Yuan et al. (2001)[25] evaluated the relationship between periodontal disease, periodontopathogens and diabetes mellitus, considering diseases and healthy sites. The results showed no significant differences in clinical analysis and prevalence of these microorganisms. However, the microorganisms tested, except *Aa*, were significantly higher in diseased sites than in the healthy sites in both groups suggesting that its play important roles in the periodontitis of both diabetics and non-diabetics. The aims of this study were to identify by PCR the presence of *A. actinomycetemcomitans*, *P. gingivalis* and *Tannerella forsythia*, and four different species of *Candida* (*C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. tropicalis)* in periodontal pockets and furcation sites of insulin-dependent diabetics and non-diabetics patients with generalized chronic periodontitis.

2. Materials and methods

This study protocol was approved (protocol number 062/2008) by the Ethical Committee of Research, Piracicaba Dental School, State University of Campinas (UNICAMP). Individuals that have accepted to participate in the study were submitted to a periodontal examination and samples collection.

2.1. Inclusion and exclusion criteria

Patients resident in Piracicaba, São Paulo, Brazil with age ranging between 31 to 68 years presenting generalized chronic periodontitis, with medical diagnosis proving health or the presence of controlled insulin-dependent type 2 diabetes mellitus, were included in the study. Exclusion criteria were the use of antibiotics and periodontal treatment during the previous 6 months, pregnancy, smoking, systemic disease, immunodepression, use of partial and/or total prosthesis, orthodontic apparatus or any medication that could affect the periodontium.

2.2. Study population

Twenty subjects of the Graduate Clinic of the Piracicaba Dental School, with a clinical diagnosis of generalized chronic periodontitis defined by PD ≥ 5 mm in ≥ 10 teeth, radiographic bone loss ranging from 30% to 50%, and ≥ 20 teeth were included. The subjects were divided into two groups: an experimental group diagnosed with insulin-dependent type 2 diabetes mellitus (n=10; DM group) and healthy control subjects without diabetes (n=10; NDM group). DM subjects reported the number of years since their diagnosis and insulin initiation and their daily consumption of insulin. For both DM and NDM were evaluated the following clinical parameters: PD (probing depth), gingival recession (GR), clinical attachment level (CAL), plaque index (PI) and gingival index (GI) by a single examiner with a periodontal probe at four sites per tooth at all teeth, excluding third molar. All clinical analysis is described in Cruz et al. (2008)[18]**.** After clinical measurements, the supragingival biofilm was removed with sterile gauze and subgingival samples were taken from the sites with the deepest PD ≥ 5 mm and with furcations in each subject using a sterile periodontal curette. Pooled biofilms from each site were separated in microtubes containing reduced transport fluid (RTF) and were stored at -20°C to be analyzed microbiologically by polymerase chain reaction (PCR).

2.3. Microbiologic assessment – PCR assays

DNA was extracted using a protocol described originally by Doyle and Doyle (1990) [26] with some modifications (Nascimento et al., 2004) [27], quantified in spectrophotometer at 260 nm (Genesys 10UV, Rochester, NY,USA), in order to obtain a standard concentration of 100 ng/mL and stored at – 20°C for subsequent PCR reactions. Briefly, samples were submitted to a lysing solution (extraction buffer and proteinase K) and then purified by chloroform: isoamil-alcohol, followed by DNA precipitation with isopropanol and ethanol 70%. The DNA was ressuspended in TE buffer (10 mM Tris-HCL, 0.1 Mm EDTA, pH 7.5) added 10ug /mL RNAse). Microbial molecular identification was carried out by PCR with specific primers for *A. actinomycetemcomitans* (Aa), *P. gingivalis* (Pg), *T. forsythia* (Tf), *C. albicans* (Ca), *C. dubliniensis* (Cd), *C. glabrata* (Cg) and *C. tropicalis* (Ct) (Table 1). Purified DNA from these microorganisms was used as a positive control. The molecular mass ladder (100 bp DNA ladder, Gibco, Grand Island, NY, USA)

was included for running in the agarose gel. PCR amplification was performed with a GeneAmp PCR system 2400 (Perkin-Elmer-Applied Biosystems) under thermal conditions showed in Table 1 specific for each pair of primers. All PCR amplification was preceded for an initial denaturation step at 94-95^{o}C for 1 to 2 min and a final elongation step at 72^{o}C for the same time. The PCR products were separated by electrophoresis in 2% agarose gels and Tris-borate-EDTA running buffer (pH.8.0). The DNA was stained with 0.5µg of ethidium bromide/mL and visualized under UV illumination (Pharmacia LKB-MacroVue, San Gabriel, CA, USA). Photo shots of the images were taken (Image Mater- LISCAP, VDS, Pharmacia Biotech Piscataway, NJ, USA) and analyzed.

2.4. Statistical analysis

The statistical analysis was performed using software SPSS Statistics 17.0 (IBM Inc., Chicago, Ill., USA). For results, the percentages of sites with Aa, Pg, Tf, Ca, Cd, Cg and Ct were compared between DM and NDM groups applying chi-square test. Differences were considered significant when $p \leq 0.05$.

3. Results

All clinical results obtained from these patients are showed in Cruz et al. (2008) [18]. The prevalence of periodontal pockets (PD ≥ 5mm) with Aa, Pg, Tf, Ca, Cd, Cg and Ct is show in Figure 1. A significant difference between DM and NDM was observed for Tf, Ca, Cd, Cg and Ct. Considering furcation sites, Figure 2 shows the percentage of Aa, Pg, Tf, Ca, Cd, Cg and Ct. Statistical difference between DM and NDM was observed for Tf, Cd, Cg and Ct. Prevalence of Pg was similar between the groups and Tf was less frequent in DM. The frequency of Aa was variable in the different periodontal sites, for both groups. Eighteen (85 %) of 20 patients (10 DM and 7 NDM) were colonized by species of *Candida* in one or more of sites evaluated. In DM, all tested *Candida* spp. (*C. albicans*, *C. dubliniensis, C. tropicalis* and *C. glabrata)* were found in periodontal pockets and furcation sites. *C. dubliniensis* was the specie more prevalent in the subgingival biofilm of periodontal pockets and furcation sites of DM. In NDM, it was detected only *C. albicans*

and *C. dubliniensis* and *C. albicans* was the most prevalent. *Candida* ssp. colonization was significantly higher in DM patients compared with NDM, both with generalized chronic periodontitis. Association was found for *C. albicans* and *C. dubliniensis* in 38.96% of periodontal pockets and 7.79% of furcations of DM patients with periodontal disease. This association was not found in patients without diabetes mellitus (Figure 3).

4. Discussion

Considering the prevalence of periodontal pathogens, our study evaluated the presence of *P. gingivalis*, *A. actinomycetemcomitans* and *T. forsythia* in subgingival biofilm of periodontal pockets and furcation sites. There was no difference for Pg and Aa between diabetics and non-diabetics patients. Tf was less frequent in diabetic patients in both periodontal sites. Yuan et al. (2001) [25] detected *A. actinomycentemcomitans, P. gingivalis, Eikenella corrodens, Treponema denticola* and *C. albicans* in subgingival plaque by PCR and evaluated periodontal parameters, comparing non-insulin dependent diabetic to non-diabetic patients. The results showed no significant differences in clinical analysis and prevalence of these microorganisms. However, when evaluated these microorganisms in diseased and healthy sites, all microorganisms tested, except Aa, was significantly higher in disease sites, for both groups, showing that periodontal conditions is more related to prevalence of these microorganisms than the presence of diabetes mellitus. The frequency of Aa in this present study was reduced in relation to other periodontal pathogens, regardless to the systemic condition. Studies have showed that Aa is more related to aggressive forms than chronic periodontitis. [5,6,25] Although the majority of the periodontal microbiota is commensal, a subset of likely opportunistic pathogens fulfills the epidemiologic requirements needed in order to be ascribed as risk/causative factors. [5]

It has also been hypothesized that the presence of a high concentration of salivary glucose combined with a reduced salivary secretion rate enhances the growth of yeasts and their adherence to epithelial oral cells in type 2 diabetes mellitus patients, [29] probably by the increase of enzymatic activity. In this study, the frequency of yeasts *Candida* in subgingival biofilm in DM and NDM patients was analyzed. The results showed that DM had higher prevalence of *Candida* spp. when compared to NDM ($p<0.05$, chi-square test). *C. glabrata*

and *C. tropicalis* were not found in NDM. In DM, *C. dubliniensis* were detected in 100% of the periodontal pockets, following by *C. albicans* (80%), *C. tropicalis* (30%) and *C. glabrata* (20%). In molar furcation sites, the prevalence of *C. albicans*, *C. dubliniensis*, *C. tropicalis* and *C. glabrata* were respectively 40%, 90%, 40% and 10% for diabetics. *C. albicans* and *C. dubliniensis* were found in association in the total of 30 periodontal pockets (38.96%) and 6 furcations (7.79%). There was no association of yeasts species in sites of NDM. When compared with periodontal bacteria detected in this study, *Candida* spp. was more prevalent in both periodontal pockets and furcation sites in diabetics. Species of *Candida*, especially *C. albicans*, have been recovered from periodontal pockets (7.1–19.6 %) of patients with chronic periodontitis.[6,13] Urzúa et al. (2008)[10] used phenotypic and genotypic methods to analyze the composition of yeast microbiota in the mucosa and subgingival sites of healthy individuals and in patients with aggressive and chronic periodontitis. Although the profiles of the species present in the mucosa of the 3 groups varied, they n noted that only *C. albicans* and *C. dubliniensis* were capable of colonizing periodontal pockets in patients with chronic periodontitis, and that only *C. albicans* was identified in the subgingival pockets of healthy individuals and patients with aggressive periodontitis. Immunosuppressive conditions such as AIDS and diabetes mellitus increase host susceptibility to infections. Portela et al., (2004)[30] and Feller and Lammer (2008)[31] observed an increase in the prevalence of *Candida* species in the oral cavities and specifically in the subgingival biofilm of HIV-seropositive patients. As mentioned previously, Yuan et al. (2001)[25] verified the presence of *C. albicans* in subgingival biofilm of diabetic patients, mainly in diseases sites, in lowest levels in comparison to other periodontal bacteria, contrasting with our results.

The detection of fungi in the subgingival region has been point out to contribute for pathogenesis of the periodontal disease and to increase the candidemia possibility, mainly in cases of immune depression.[13,14] However, the role of yeasts, mainly *Candida albicans*, in chronic periodontitis is yet unclear.[22] The involvement of fungi in the pathogenesis of periodontal disease has been considered due to the ability of this microorganism has to adhere to epithelial cells and penetrate in connective tissues thus causing an inflammatory reaction.[32] The results reported in this work, indicate that there are differences in the

prevalence of the yeasts recovered from subgingival samples in patients with DM, when compared to NDM. *C. albicans* and *C. dubliniensis* seem to have a preferential capacity to colonize the periodontal pockets of diabetics patients; however it can also be found in non-diabetic patients. The presence of *Candida* species and their possible role in the development of chronic periodontitis and/or the exacerbation of the periodontal condition in diabetic patients remain unclear and that should be further studied.

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Table 1. Primers used in PCR analysis

	Primer sequence	Size of PCR product	Thermal conditions	References
Aa	5´-AAACCCATCTCTGACTTCTTCTTC-3´ 5´- ATGCCAACTTGACGTTAAT- 3´	557bp	36 cycles - 95^{o}C for 30s, 60^{o}C for 1min and 72^{o} C for 1 min	Ashimoto et al., 1996
Pg	5´- AATCGTAACGGGCGACACAC-3´ 5´- GGGTTGCTCCTTCATCATAC-3´	593bp	30 cycles - 94°C for 1 min,70°C for 1 min and 72°C for 1 min	Benkirane et al., 1995
Tf	5´- GCGTATGTAACCTGCCCGCA-3´ 5´-TGCTTCAGTGTCAGTTATACCT-3´	641bp	36 cycles - 95°C for 30s,60°C for 1 min and 72°C for 1min	Slots et al., 1995
Ca	5´- ACTGCTCAAACCATCTCTGG-3´ 5´- CACAAGGCAAATGAAGGAAT-3´	452 bp	38 cycles - 94°C for 1 min,53°C for 1 min and 72°C for 30 s	This study
Cd	5´- GTATTTGTCGTTCCCCTTTC-3´ 5´- GTGTTGTGTGCACTAACGTC-3´	288 bp	38 cycles - 94°C for 1 min,54°C for 1 min and 72°C for 30 s	Donnelly et al., 1999
Ct	5´- CACCCAAACAATTACCAAGT-3´ 5´- TGCAAACTCTTTACCTGGAT-3´	253 bp	36 cycles - 94°C for min,51°C for 1 min and 72°C for 30 s	This study
Cg	5´- GGAGATAGACTGGGCGTTAT-3´ 5´- GTTGTTCAATGGCTTTCTTC-3´	314 bp	30 cycles - 94°C for 1 min,56°C for 1 min and 72°C for 30 s	This study

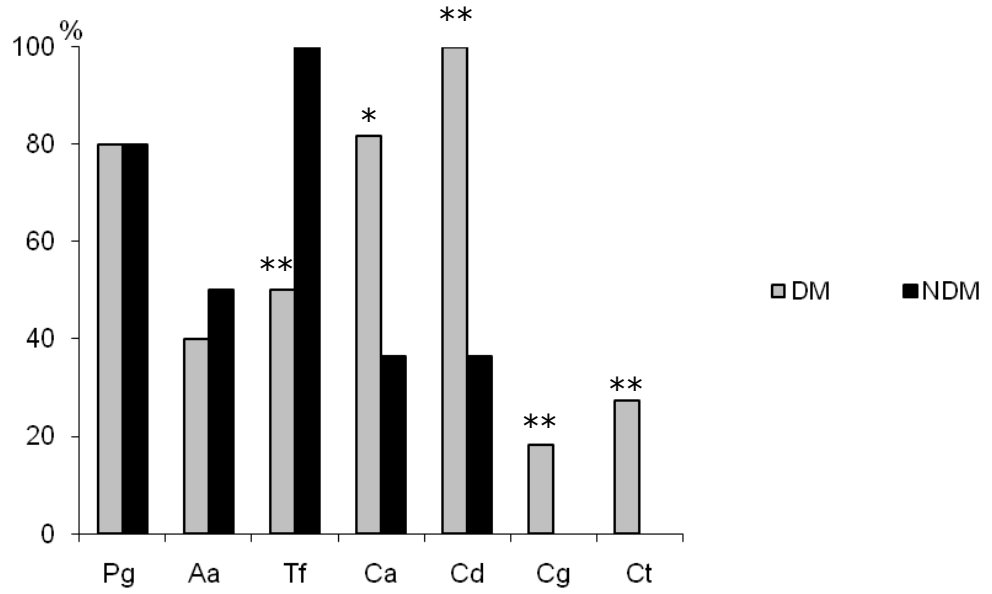

Figure 1. Prevalence of microorganims found in subgingival biofilm of periodontal pockets from type 2 diabetes mellitus (DM) or non-diabetes mellitus patients (NDM)

* p<0.05; **p<0.01 – when diabetic and non-diabetics patients were compared using x^2 test.

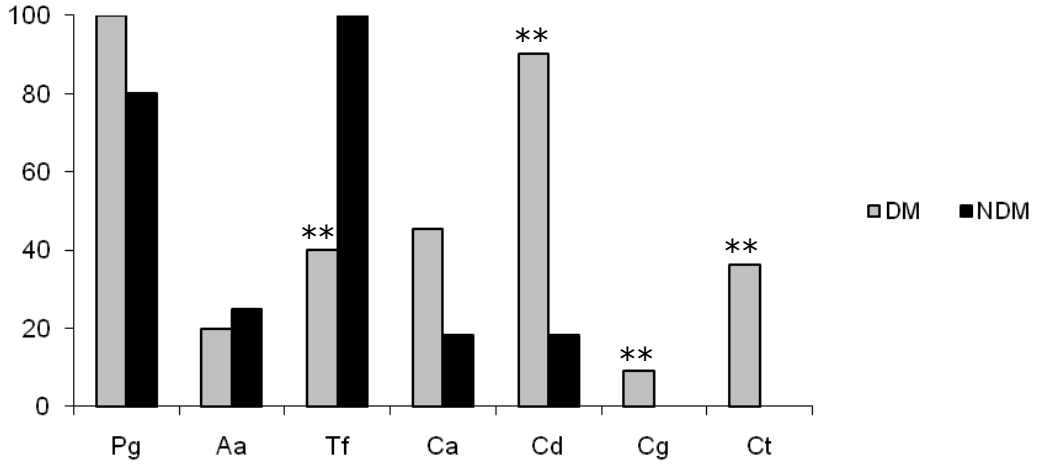

Figure 2. Prevalence of microorganims found in subgingival biofilm of furcation sites from type 2 diabetes mellitus (DM) or non-diabetes mellitus patients (NDM).

**p<0.01 – when diabetic and non-diabetics patients were compared using x^2 test.

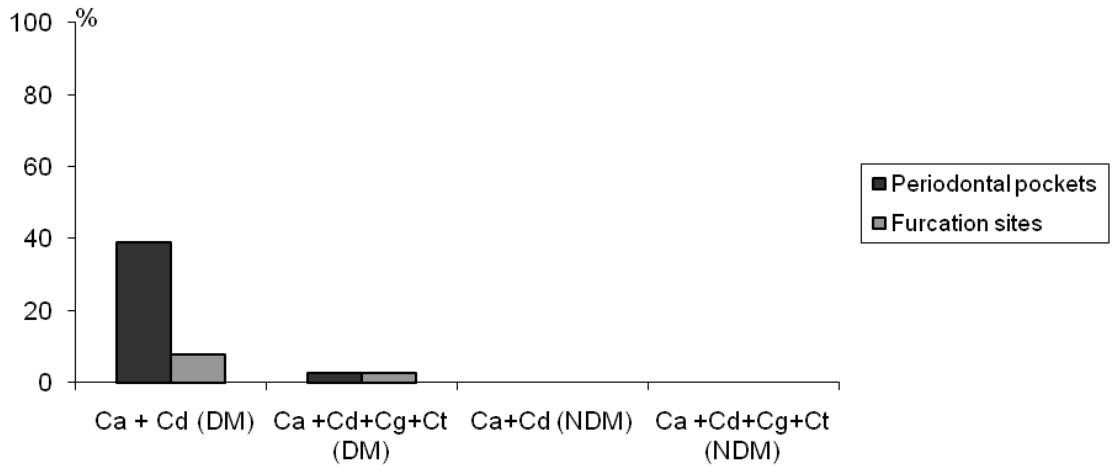

Figure 3. Association between *Candida* spp. in periodontal pockets and furcation sites of type 2 diabetes mellitus (DM) and non-diabetes mellitus (NDM) patients.

5. CAPÍTULO 3

Genetic and phenotypic evaluation of *Candida albicans* strains isolated from subgingival biofilm of diabetic patients with chronic periodontitis

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Abstract

Candida spp. are commensal microorganisms that inhabit different sites of the oral cavity. In healthy subjects, who have intact immunological system, these yeasts do not cause disease. However, in immunosuppressive conditions, such as diabetes mellitus, species of *Candida* can express different virulence factors and consequently to become pathogenic. Studies have detected the presence of *Candida* spp. in periodontal sites of patients with chronic periodontitis, especially when they are immunologically compromised. But it is still unknown the role of these microorganisms in the pathogenesis of periodontal disease. The objectives of this study were: 1) to isolate and identify *Candida albicans* strains from subgingival sites of diabetic patients with chronic periodontitis; 2) to evaluate the following virulence factors: colony morphology, proteinase, phospholipase and haemolysin activities and cell surface hydrophobicity (CSH) in different atmospheric conditions and 3) to determine genetic patterns of these *C. albicans* isolates. Microbial samples were collected from subgingival sites and seeded in CHROMagar for subsequent identification of *C. albicans* by PCR. For the phenotypic tests, all strains of *C. albicans* were grown under two atmospheric conditions: reduced oxygen (RO) and anaerobiosis (ANA). Genetic groups (genotypes) were defined based in the identification of the transposable introns in the 25S rDNA by PCR. The results obtained for tested virulence factors were analyzed according to the atmospheric condition or genetic group, using chi-square and Wilcoxon non-parametric tests. In this study, 128 strains were identified as *C. albicans* and of these, 51.6% were genotype B and 48.4% were genotype A. Genotype C was not found in this present study. The most of strains were alpha-hemolytic in both atmospheric conditions, without statistical difference. However, when compared the genotypes, 46.1% of the genotype A strains were beta-hemolytic. In relation to colony morphology, 100% of the strains under ANA showed rough colonies, whereas in RO, most of them had smooth colony. The rough aspect was prevalent for genotype A. In regard to the proteinase and phospholipase activities, strains of *C. albicans* did not produce these enzymes in the total absence of oxygen. In RO, most of strains had high proteinase activity and were positive for phospholipase tests ($p<0.05$). Hydrophobicity was higher in anaerobiosis and mainly for the genotype A. In conclusion, oxygen concentration in the environment influences the virulence factors of *C. albicans* strains isolated from subgingival sites of diabetic and periodontal patients and the genotype A seems to be more virulent, considering the phenotypic tests evaluated in this study.

Keywords: periodontal disease, diabetes mellitus, *Candida* spp., virulence factors

1. Introduction

Candida species are microorganisms that live commensally in the oral cavity of healthy individuals but their incidence and virulence seems to be increased in immunocompromised individuals (Arendorf et al., 1980; Belazi et al., 2005). These species can become pathogenic in response to physiological changes in the host causing oral candidiasis or invasive systemic infection (Jabra-Rizk et al., 2001). Mucosal surfaces are the primary oral body reservoirs of these yeasts, but they can also be found in dental plaque, endodontic infections, peri-implantitis lesions and subgingival biofilm of periodontal pockets from different forms of periodontitis, especially in HIV-positive and diabetic patients (Velegraki et al., 1999; Baumgartner et al., 2000; Reynaud et al., 2001; Yuan et al., 2001; Portela et al., 2004; Song et al., 2005). The higher prevalence of *Candida* spp. in diabetic patients have been associated to type and duration of disease and the degree of glycaemic control (Darwazeh et al., 1990; Willis et al., 2000).

Besides, *Candida* ssp. may produce some virulence factors that facilitate their proliferation and consequently adherence to the epithelium and invasion to the connective tissue. These yeasts produce essentials exoenzymes that improve their capacity to establish itself as a colonizing and/or infectious microorganism (Willis et al., 2001). The exoenzymes, such as secreted aspartyl proteinase (SAPs) and phospholipases, degrade immunoglobulins and proteins from the extracellular matrix, inhibit phagocitosis of polymorphonuclear neutrophils and induce inflammatory reactions (Reynaud et al., 2001; Hube et al., 2001). Another virulence factor is related to iron acquisition by *Candida* spp. Iron is an essential nutrient for many microorganisms and its uptake may play a special role in promoting infections (Ramanan and Wang, 2000). The secretion of haemolysin, followed by iron acquisition, facilitates hyphal invasion and the development of disseminated candidiasis. An accentuated blood glucose concentration may contribute, directly or indirectly, to increase haemolysin activity among *C. albicans* isolates in diabetic patients (Tsang et al., 2007).

Adherence to host tissue is the first step in the pathogenic process. The mechanisms of adherence to different cellular types or surfaces by *Candida* ssp. are complex. This

process is achieved by a combination of specific (receptors) and nonspecific mechanisms (electrostatic forces, aggregation and cell surfaces hydrophobicity - CSH) (Manfredi et al., 2006). CSH has a central role in the pathogenesis of *C. albicans*. Hydrophobic cells exhibit greater adherence to epithelial and endothelial cells and extracellular matrix proteins and seems to be more resistant to killing by phagocytes (Hazen and Glee, 1995). *Candida albicans* is unique among *Candida* species in that CSH status varies in response to different environmental conditions and growth phases (Hazen et al., 2001).

Another property of *Candida* sp. is the ability to grow either aerobically or anaerobically. These microorganisms developed adaptative mechanisms to survive in both situations. Oxygen can generate reactive products during an infection and induce oxidative stress response. Treatment of *C. albicans* with low concentrations of superoxide generating agents, such as hydrogen peroxide, induces a redox potential with the activation of antioxidant enzymes which protects cells from the lethal effects of a subsequent challenge with higher concentrations of these oxidants (Jamieson et al., 1996). Another situation is anaerobic environment such as root canal systems and periodontal pockets that leads to polymicrobial infections. Rosa et al. (2008) demonstrated a increment in SAP secretion when *C. albicans* strains, recovered from periodontal pockets and other intra-oral sites, were grown under anaerobic conditions, suggesting that the oxygen concentration in the atmosphere surrounding cells can exert influence on the virulence attributes of *C. albicans.*

Besides the physiological factors, genotypic diversity, assessing by different molecular typing techniques, have become fundamental for studying the epidemiology of *Candida* isolates. Pizzo et al. (2002) suggested that heterogenotype within subgingival *C. albicans* isolates is not just the result of the spreading of *Candida* microorganisms from saliva or biofilm, but also new strains adapted to subgingival pockets that can develop different virulence properties. This study evaluated some virulence factors of *C. albicans* isolated from subgingival biofilm of patients with chronic periodontitis with insulin-dependent type 2 diabetes mellitus and investigated the relationship with different microenvironmental conditions and genetic diversity.

2. Material and methods

This research was approved by the ethical committee in research of the Piracicaba Dental School, State University of Campinas, SP, Brazil.

2.1 Inclusion and exclusion criteria

Patients resident in Piracicaba, São Paulo, Brazil with age ranging between 31 to 68 years with chronic periodontitis (CP) and medical diagnosis of type 2 diabetes mellitus were included in the study. Glycemic control with insulin supplementation was confirmed by an endocrinologist. Exclusion criteria were: use of antibiotics and periodontal treatment during the previous 6 months, pregnancy, smoking, systemic disease, immunodepression, patients with clinical manifestation of oral candidosis, use of partial and/or total prosthesis, use of orthodontic apparatus or any medication that could interfere with the periodontium or the response to periodontal therapy.

2.2 Patient Selection

Eleven patients with clinical diagnosis of generalized chronic periodontitis defined by probing depth (PD) ≥ 5 mm in ≥ 10 teeth, radiographic bone loss ranging from 30% to 50%, and ≥ 20 teeth were included. After clinical analysis, the supragingival biofilm was removed with sterile gauze. Subgingival samples were taken from the sites with the deepest PD ≥ 5 mm in each subject using a sterile periodontal curette. Pooled biofilms from each site were separated in Eppendorf® microtubes containing 1mL of reduced transport fluid (RTF). Immediately after collecting, the samples from each site were diluted and plated onto a Sabouraud Dextrose Agar (SDA) with Cloranfenicol and chromogenic medium (CHROMagar Candida®, Biomerieux, Paris, France) and incubated at 37°C during 48 h in reduced oxygen atmosphere (10% CO_2 and 90% of air). The green colonies grown on the agar plate were randomized selected and cultures stored in glycerol stock at -20^{o}C for posterior identification by PCR.

2.3. PCR (polymerase chain reaction)

DNA from the *Candida* isolates was extracted using a protocol described by Nascimento et al. (2004), quantified in spectrophotometer at 260 nm (Genesys 10UV, Rochester, NY,USA), in order to obtain a standard concentration of 100 ng/mL and stored at – 20°C for subsequent PCR reactions. DNA samples were identified by PCR using specific primers for the portion corresponding to the gene AAT1a (ID 3643468) (F: 5 'ACT GCT CAA ACC ATC TCT GG -3 ' and R: 5 'CAC AAG GCA AAT GAA GGA AT - 3 with fragment size of 472bp) of *C. albicans.* Purified DNA from *C. albicans* (ATCC 90028) was used as a positive control. The molecular mass ladder (100 bp DNA ladder, Gibco, Grand Island, NY, USA) was included for running in the agarose gel. PCR amplification was performed with a GeneAmp PCR system 2400 (Perkin-Elmer-Applied Biosystems) under thermal conditions: 72^{o} C for 5 min, 30 cycles of 95^{o} C for 30 s, 55^{o}C for 45s and 72^{o} C for 30 s and extension at 72^{o}C for 5 min. The PCR products were separated by electrophoresis in 2% agarose gels and Tris-borate-EDTA running buffer (pH.8.0). The DNA was stained with 0.5ug of ethidium bromide/mL and visualized under UV illumination (Pharmacia LKB-MacroVue, San Gabriel, CA, USA).

2.4. Colony morphology

Colony morphology was evaluated for all *C. albicans* isolates, using a blood plate assay. Media were prepared by adding 7 mL fresh sheep blood to 100 mL SDA (Merck) supplemented with glucose at a final concentration of 3% (w/v). The final pH of the medium was approximately 5.6. *C. albicans* isolates were grown on SD broth for 24 h and an inoculum of 10^8 CFU/mL (absorbance 0.5 in sterile saline at 600nm) was spread in the blood plates. These plates were incubated at 37°C in reduced oxygen atmosphere (10% CO_2 and 90% air – Water-Jacked CO2 Incubators-Cole Parmer Instruments, EUA) or anaerobiosis (10% CO_2, 10% H_2 and 80% N_2 – Anaerobic Workstation, Don Whitley Scientific, Shipley, UK) for 48 and 72h, respectively. After this, the colony morphology was distinguished in smooth or rough using a stereoscopic microscope.

2.5. Proteinase and phospholipase activity determination by the agar plate method

All *C. albicans* isolates were tested, in triplicate on three independent experiments, to verify the enzymatic activity of proteinases (SAPs) and phospholipases (Willis et al., 2001; Price et al., 1992). The test medium for proteinases was BSA (bovine serum albumin) agar medium containing 2g of BSA, 145g of YNB (Yeast Nitrogen Base - Difco Laboratories, Detroit), 20g of glucose and 20g of agar per liter of distilled water. The test medium for phospholipases consisted of 10g of peptone, 57.3g of sodium chloride, 0.55g of calcium chloride, 30g of glucose, 20g of agar, and 100 mL of 50% sterile egg yolk (egg yolk enrichment) per liter of distilled water. Test isolates were grown on SDA for 24 h, and an inoculum of 10^8 CFU/mL in sterile saline (absorbance 0.5 at 600nm) was transferred to the test medium. The plates were incubated at 37°C in reduced oxygen atmosphere or anaerobiosis during 48h and 72h for proteinases and phospholipases, respectively. The enzymatic activity was determined by the formation of a halo around the yeast colony, and measured in terms of the ratio of the diameter of the colony to the total diameter of colony plus zone of precipitation (Pz), regarding to the method described by Price et al., 1982. According to this system, Pz = 1,0 means that the test strain is negative for proteinase/phospholipase, while a value of $Pz \leq 0.63$ signifies that the test strain is releasing large amounts of proteinases/phospholipases (strongly positive). Values of Pz of between 0.64 and 0.99 signify that the test strain is releasing small amounts of proteinases/phospholipases (positive).

2.6. Determination of haemolysin activity

Haemolysin activity was evaluated for all *C. albicans* isolates, using a blood plate assay (Tsang et al., 2007). Media were prepared by adding 7 mL fresh sheep blood to 100 mL SDA (Merck) supplemented with glucose at a final concentration of 3% (w/v). The final pH of the medium was approximately 5.6. Test isolates were grown on SDA for 24 h, and an inoculum of 10^8 CFU/mL in sterile saline (absorbance 0,5 at 600nm) was transferred to the test medium. The plates were then incubated at 37°C in reduced oxygen or anaerobiosis for 48 h and 72h, respectively. A further 10 µL of saline but without yeast cells was overlaid onto the same plate. A reference strain of *C. albicans* (ATCC 90028) was used as a positive control. In addition, one standard strain of *Staphylococcus aureus*

(ATCC 10832) and one for *Streptococcus mutans* (UA159) which induce beta and alpha haemolysis were used as positive controls. Gama hemolytic strains (γ – hemolytic) do not produce halo, alpha hemolytic (α – hemolytic) strains produced green halo and beta hemolytic (β - hemolytic) strains produced a yellow halo. The assays were conducted in triplicate on three independent experiments for each yeast isolate tested.

2.7. Cellular Superficial Hydrophobicity Assay (CSH)

Hydrophobicity assay (CSH) was evaluated according Rodrigues et al. (1999) for all *C. albicans* isolates, in two independent experiments. Fifty mL of Sabouraud Dextrose Broth (Difco Laboratories Detroit, MI) was inoculated with yeast cells and incubated overnight at 37°C in 10% reduced oxygen or anaerobiosis. Briefly, yeast cells were harvested washed twice in phosphate buffer, pH 8.0. A yeast suspension was prepared in the same buffer, to hold an optical density (A0) of 0.4 – 0.6 (at 600 nm). 150 µL of hexadecane was added to 3 mL of this yeast suspension. After 10 min of the incubation at 30°C, the tubes were vortex twice for 30 seconds. After allowing phase separation for 30 min, the optical density of the lower aqueous phase (A1) was measured and compared with that obtained prior to the mixing procedure (A0). The percentage of cells in the hexadecane layer (adhered cells) was used to estimate the hydrophobicity, using following formula: % H= A0 – A1/ A0 x 100%.

2.8. Genotyping

Ribosomal sequences are extensively used for genotyping of many fungal pathogens. The method developed by McCullough et al. (1999), uses a pair of primers designed to span the region that includes the site of the transposable group 1 intron of the 25S rRNA gene (rDNA), and classify *C. albicans* strains into three genotypes, according to size of PCR products: genotype A (approximately 450 bp), genotype B (approximately 840 bp), and genotype C (two products: approximately 450 and 840 bp). DNA of the yeasts previously extracted and spectrophotometrically quantified (100 ng/mL) was submitted to PCR reactions. PCR was performed using the primers CA-INT-L (5’ ATA AAG GGA AGT CGG CAA ATA GAT CCG TAA – 3’) and CA – INT- R (5’ CCT TGG CTG TGG

TTT CGC TAG ATA GTA GAT – 3'). The products were analyzed by electrophoresis through 3% (wt/vol) agarose gel. Bands were visualized by UV transllumination after ethidium bromide staining.

2.9. Statistical analysis

The results obtained for tested virulence factors (enzymatic activities and CSH) were analyzed according to the atmospheric condition or genetic group, using chi-square and Wilcoxon non-parametric tests.

3. Results

3.1. Identification of C. albicans by PCR

In this study, 172 *Candida* spp. strains were obtained from subgingival biofilm of patients with chronic periodontitis and controlled insulin-dependent type 2 diabetes mellitus, and isolated in CHROMagar. From these, 128 were identified as *Candida albicans* by PCR. *C. albicans* strains were identified in 63,63% of periodontal sites from 11 patients with diabetes mellitus and chronic periodontitis

3.2. Virulence factor x atmospheric conditions

3.2.1. Colony morphology

In relation to colony morphology, 100% of the strains under anaerobiosis showed rough colonies, whereas in reduced oxygen, most of them (71.9%) had smooth colony (Table 1), showing statistical difference between the microenvironmental condition.

3.2.2. Proteinase and phospholipase activities

All *C. albicans* isolates showed proteinase/phospholipase activity in oxygen reduced conditions but not in anaerobiosis. Pz values for proteinase tests were ranged from 0.29 to 1.0 for the isolates incubated in reduced O_2. In this microenvironmental condition, only 2.4% of the strains did not produce these enzymes, 3.1% were positive and the vast

majority (94.5%) was strongly positive for the production of enzyme proteinase. *C. albicans* showed different activities of phospholipases with Pz ranging from 0.41 to 1.0 in reduced oxygen atmosphere, presenting 8.6% of the strains without phospholipase activity (negative), 58.3% having low activity (positive) and 33.1% high activity (strongly positive). The strains were not able to produce the phospholipase enzyme in anaerobic (Table 1). These enzymatic activities were statistically different comparing the microenvironmental condition (reduced O_2 or anaerobic).

3.2.3. Haemolysin activity

For both atmosphere conditions, the results obtained were: 1.6% γ – hemolytic; 64.40% α – hemolytic; 34.30% β – hemolytic, showing that this variable did not influence in the haemolysin activity for tested *C. albicans* strains (Table 1).

3.2.4. Cellular Superficial Hydrophobicity Assay (CSH)

Hydrophobicity was classified in three levels: low (0-30%), moderate (31-59%) and high (above 60%). In reduced oxygen atmosphere, the results obtained were 34.40% for low hydrophobicity, 43.70% for moderate and 21.90% to high hydrophobicity. To anaerobiosis, the results were 13.47% for low hydrophobicity, 34.56% for moderate and 51.97% to high hydrophobicity (Table 1). Yeast cells grown under anaerobic conditions were more hydrophobic than when grown in reduced oxygen condition, with statistical difference between the microenvironmental conditions ($p<0.05$, chi-square test).

3.3. Genotyping

A total of 128 strains isolated from patients with chronic periodontitis diabetics were submitted to molecular typing by the method described above. 51.6% were genotype B and 48.4% were genotype A. Genotype C was not found in this present study.

3.3.1. Virulence factors x genotyping

Genotype A – In reduced oxygen atmospheric condition, 95.2% and 51.6% were strongly positive for proteinase and phospholipase activities, respectively. 51.6% of *C.*

albicans strains was α – hemolytic, for both conditions. Smooth morphology was observed in 59.7% of strains with genotype A. For CSH, 61.3% of strains had high hydrophobicity in anaerobiosis and 37.1% in reduced oxygen (Table 2). Statistical difference was observed for all virulence factors, except for hemolysis ($p<0.05$, chi-square test).

Genotype B - In reduced oxygen atmospheric condition, 95.5% and 15.6% were strongly positive for proteinase and phospholipase activities, respectively. 81.8% of strains were only positive for phospholipase activity. 75.8% of *C. albicans* genotype B was α – hemolytic, for both conditions. Smooth morphology was observed in 83.3% of strains. For CSH, 41.5% of strains had high hydrophobicity in anaerobiosis and only 7.6% in reduced oxygen atmosphere (Table 2). Statistical difference was observed for all virulence factors except for hemolysis and CSH ($p<0.05$, chi-square).

When genotypes A and B were compared, there was statistical difference between them for phospholipase activity in reduced oxygen and CSH in anaerobiosis.

4. Discussion

Candida is present as a commensal in the oral cavities of up to 40% of healthy individuals. The number of organisms in the saliva of carriers increases in pregnancy, with tobacco smoking, and when dentures are worn (Ellepola and Samaranayake, 2000). The prevalence of *Candida* species in oral cavity of immunosuppressed individuals has been found higher in comparison to healthy population. Peterson et al. (1997) observed that the prevalence of oral yeasts from saliva of hospitalized patients was 55%. In patients with advanced cancer, this prevalence ranged between 47% and 87%. In diabetic patients, the presence of *Candida* spp. in oral mucosa reached up to 80% (Soysa et al., 2006). Few studies have demonstrated the presence of *Candida* spp. in periodontal pockets. Barros et al. (2008) found 39.6% of periodontal sites with the presence of *C. albicans* in healthy patients. In this present study, *C. albicans* strains were identified in 63,63% of periodontal sites from patients with diabetes mellitus and chronic periodontitis.

Virulence factors of *Candida* species may also be involved in the pathogenesis of several oral diseases. However, few studies have investigated their role in host colonization and development of infection (Koga- Ito et al., 2006). Enzymatic secretion from oral

isolates of *C. albicans*, especially phospholipases (PLs) and secreted aspartyl proteinases (SAPs), is determinant not for only commensal colonization but also for pathogenic potential of such yeasts (Sugita et al., 2002; Willis et al., 2000). Studies have reported that 30 -100% of the oral isolates of *C. albicans* produce phospholipases, with variable degrees of enzymatic activity (Hannula et al., 2001; Barros et al., 2008). Phospholipase activity was detected in 91.4% of *C. albicans* isolates in this study when incubated in reduced oxygen atmosphere. Enzymatic activity may depend on the site where the pathogen was isolated; for example, phospholipase activity has been found in 100, 55, 50 and 30% of the *Candida* species isolated from chronic periodontitis, blood, wound infections and urine, respectively (Price et *al*., 1982; Barros et al., 2008). Phospholipase expression has shown to be affected by growth conditions (Samaranayake et al., 2006). It has also been hypothesized that the presence of a high concentration of salivary glucose combined with a reduced salivary secretion rate enhances the growth of yeasts and their adherence to epithelial oral cells in type 2.

Considering proteinase production, Koga – Ito et al. (2006) showed that SAPs activity is significantly higher in denture wearers with signs of candidiasis than in denture wearers with a normal palatal mucosa. Another study has demonstrated that proteinase expression is not significantly higher in *Candida* isolates of patients with diabetes compared to healthy patients, and that type 2 diabetes mellitus patients have higher proteinase levels than type 1 (Manfredi et al., 2006). In our study, proteinase activity was detected in 94.5% of the *C. albicans* isolates when incubated in reduced oxygen atmosphere. Our findings are agreement to data obtained from Tsang et al., 2007, that found high proteinase activity in type 2 diabetes mellitus patients. In this present study, there was no activity of phospholipase or proteinase in anaerobic conditions. Rosa et al. (2008) evaluated strains of *C. albicans* isolated from healthy patients with chronic periodontitis and observed an increase on SAPs activity in anaerobiosis compared to aerobiosis. Unfortunately, our results cannot be compared to those obtained in that study. Strains of *C. albicans* were isolated from healthy patients and in this present study, they belong to diabetic patients. Besides, although the atmospheric conditions seem to be

similar, we incubated in anaerobic chamber and they used anaerobic jars, showing differences in the methodology (Rosa et al. 2008).

Studies evaluating the activity of haemolysin in *C. albicans* are limited and, to the best of our knowledge, there are no studies on the activity of haemolysin of *C. albicans* isolates from periodontal pockets of diabetic patients. Manns et al., (1994) defined the conditions under which *C. albicans* can display haemolytic activity and found out that haemolysis is not observed when glucose is not available in the culture medium. In this present study, hemolytic activity was detected in 98.5% of the *C. albicans* isolates when studied in both conditions: reduced oxygen or anaerobiosis, and that, 64.3% were alpha hemolytic, 34.3% beta hemolytic and 1.5% gamma hemolytic. Luo et al. (2001) evaluated 80 *Candida* isolates from clinical sources in different geographical locates and detected only alpha haemolysis in experiments with glucose free sheep blood agar. A high blood glucose concentration may contribute, directly or indirectly, to increased hemolysin activity among *C. albicans* isolates in diabetic patients (Tsang et al., 2007).

In this study also evaluated the cell surface hydrophobicity (CSH) in reduced oxygen and anaerobiosis and the results showed that strains were more hydrophobic when grown in anaerobic conditions. The results were 51.97% for high hydrophobicity in anaerobic and 21.90% for reduced oxygen atmosphere. Hydrophobic interactions may be of importance in promoting tissue invasion by filamentous yeast cells. Germ tubes of *C. albicans* are able to adhere to fibronectin, fibrinogen, and complement via cell surface receptors (Calderone et al., 1991). CSH favors attachment of *C. albicans* to extracellular matrix components (ECM), which are intimately associated with host cells surface (Silva et al., 1995). Investigation has suggested that increase in CSH produces impairment of phagocytosis, increasing the resistance to blood clearance and consequently virulence of *Candida* species (Rodrigues et al., 1999).

The present study reports the first evaluation of the different genotypes of *C. albicans* by 25S rDNA PCR from patients with diabetes mellitus and periodontal disease. The results show that these patients are preferentially colonized with genotype B (51.6%), although published studies have shown a high incidence of genotypes A in healthy population (McCullough et al., 1999; Tamura et al., 2001). However, there are some

evidences to show an increase in the isolation of genotype B in immunocompromised patients, particularly in HIV patients (Velegraki et al., 1995; Vidotto et al., 1999). The transposable intron region of the 25S rDNA gene was found to be associated with flucytosine susceptibility and genotype B is more susceptible to flucytosine (McCullough et al., 1999).

The genotype B was prevalent but the genotype A was the most virulent in this study. For genotype A, 51.6% was α – hemolytic and 61.3% of strains had high hydrophobicity, for both atmospheric conditions. For genotype B, 75.8% of *C. albicans* was α – hemolytic for both conditions and 41.5% of strains had high hydrophobicity in anaerobiosis and only 7.6% in reduced O_2. In other study, genotype A was associated with increased resistance to antifungal (McCullough et al., 1999). This PCR method offers high levels of reproducibility and discrimination facilitating the delineation of *C. albicans* genotypes, besides to be easily performed in laboratory (Kumar et al., 2006).

In conclusion, oxygen concentration in the environment influences the virulence factors of *C. albicans* strains isolated from subgingival sites of diabetic and periodontal patients and the genotype A seems to be more virulent, considering the phenotypic tests evaluated in this study.

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Table 1. Frequency of virulence factors of *Candida albicans*, according to atmospheric condition.

Virulence factor	Results	Scores	RO (%)	ANA (%)
Colony morphology	Rough	1	21.9	100
	Less rough	2	6.3	0
	Smooth	3	71.9	0
Proteinase activity	Negative – Pz = 1	0	2.4	100
	Positive – Pz = 1- 0.63	1	3.1	0
	Strongly positive - Pz < 0,63	2	94.5	0
Phospholipase activity	Negative – Pz = 1	0	8.7	100
	Positive – Pz = 1- 0.63	1	58.3	0
	Strongly positive - Pz < 0,63	2	33.1	0
Hemolysin activity	Gama-hemolysis	0	1.6	1.6
	Alpha-hemolysis	1	64.1	64.1
	Beta- hemolysis	2	34.4	34.4
Hydrophobicity	Low (0 -30%)	1	34.4	16.7
	Moderate (31-59%)	2	43.8	31.7
	High (above 60%)	3	21.9	51.6

RO – reduced oxygen; ANA - anaerobiosis

Table 2. Frequency of virulence factors of *Candida albicans*, according to genotyping.

Virulence factor	Results	Scores	Genotype A (%)		Genotype B (%)	
			RO	ANA	RO	ANA
Colony morphology	Rough	1	35.5	100	9.1	100
	Less rough	2	4.8	0	7.6	0
	Smooth	3	59.7	0	83.3	0
Proteinase activity	Negative – Pz = 1	0	0	100	3.0	100
	Positive – Pz = 1- 0.63	1	4.8	0	1.5	0
	Strongly positive - Pz < 0,63	2	95.2	0	95.5	0
Phospholipase activity	Negative – Pz = 1	0	16.1	100	1.5	100
	Positive – Pz = 1- 0.63	1	32.3	0	83.1	0
	Strongly positive - Pz < 0,63	2	51.6	0	15.4	0
Hemolysin activity	Gama-hemolysis	0	3.2	3.2	0	0
	Alpha-hemolysis	1	51.6	51.6	75.8	75.8
	Beta- hemolysis	2	45.2	45.2	24.2	24.2
Hydrophobicity	Low (0 -30%)	1	22.6	11.7	45.5	16.9
	Moderate (31-59%)	2	40.3	25.0	47.0	41.5
	High (above 60%)	3	37.1	63.3	7.6	41.5

RO – reduced oxygen; ANA – anaerobiosis

6. CAPÍTULO 4

In vitro evaluation of adhesion and invasion of *Candida albicans* in gingival human fibroblasts.

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Abstract

Candida species are opportunistic pathogens that live commensally in the mucosa of oral cavity, but can also be found in supra and subgingival biofilm. However, their role in the periodontal disease is not known yet. The objectives of this study were to evaluate the ability of adhesion and invasion of *C. albicans* strains in gingival human fibroblasts cultures and to measure nitric oxide concentration (NO) produced by cells in the presence of these yeasts. Sixteen strains of *Candida albicans* isolated from patients with chronic periodontitis and diabetes mellitus type II were divided into two groups: highly or weakly hydrophobic, according to phenotypic tests. Primary cultures of human fibroblasts were isolated from gingival biopsies and after subsequent subcultures, the cells were seeded ($1x10^5$cells/well) into culture plates containing RPMI medium and incubated for 24 h to reach a confluent monolayer of cells. After this period, *C. albicans* strains ($1x10^3$cells/cm^2) were applied into these plates and maintained for 2 and 4h for the adhesion and invasion assays, respectively. The number of adherent or invasive yeasts was assessed by counting the colony-forming unities. The production of NO by fibroblasts in the presence of *C. albicans* was also quantified using Griess reagent after each assay. The results showed that strains with high hydrophobicity have more ability to adhere and invade fibroblasts, considering absolute number of recover cells and percentage of these events ($p<0.05$, ANOVA and Tukey). The production of NO was higher for the most hydrophobic strains, but without statistical difference when compared to other strains ($p>0.05$). These data indicated that the hydrophobicity may interfere on the ability of adhesion and invasion of *Candida albicans* in fibroblasts cultures.

Keywords: *Candida* albicans, periodontal disease, diabetes mellitus type II, hydrophobicity, human fibroblasts

1. Introduction

Candida albicans is a commensal inhabitant of the normal human microflora but can also cause a variety of infections ranging from superficial mucosal infections to haematogenously disseminated infections.[1] Mucosal surfaces are the primary oral reservoirs of these yeasts,[2] following by biofilm supra and subgingival.[3] The microbial balance among different regions inside the mouth influences the integrity of oral tissues, which may be disrupted by systemic conditions such as immunosuppressive diseases, organ transplantation and medications [4,5] that can result in host infections such as oral candidiasis. *Candida albicans* has been also isolated from periodontal pockets in different forms of periodontitis, especially HIV-positive and diabetics patients. [6,7,8] *Candida albicans* have a range of virulence factors that can be potentially relevant to the pathology of periodontal disease, such as the ability to adhere and invade gingival connective tissue.[9,10] Hydrolytic enzymes secreted by *Candida* spp. may inactive host cell surface components and immune factors during invasion.[11] One important virulence attribute of *C. albicans* is cell surface hydrophobicity (CSH) that is conferred by the mannosylated surface proteins that cover the fungal cells. Some of these proteins confer the ability to adhere to the host cells or to inanimate substrata, through the increasing of germinative forms and resistance against macrophages, which is essential to the establishment of chronic lesion. This way, *C. albicans* cells with high cell surface hydrophobicity rates present high chance of adherence to the different host tissues compared to cells with low rates of hydrophobicity.[12]

The analysis of the adherence capacity to both oral epithelial cells and cells from connective tissue would be of interest in the dissemination, infection and or persistence of *Candida* ssp. in the oral environment. Most studies have employed the exfoliated epithelial cells derived from host tissues, such as buccal cells or vaginal cells or established epithelial cell lines to determine the fungal adherence.[13] However, fibroblast cultures, primary or lineages cells have demonstrated to be a specific and reproducible model for adherence and invasion of yeasts.[14] Besides, this system is useful to study the production of several molecules by fibroblasts, such as cytokines and nitric oxide, related to progression of inflammation and tissue destruction.[15] These molecules are keys for the understanding the pathogenesis of many diseases such as the periodontitis. The objectives of this study were

to evaluate the ability of adhesion and invasion of *C. albicans* strains, isolated from periodontal pockets of periodontal patients, in gingival human fibroblasts cultures and to measure nitric oxide concentration (NO) produced by cells in the presence of these yeasts.

2. Methods

2.1. Gingival fibroblasts isolation

The procedure for establishing the primary culture of gingival fibroblasts is based on the protocol described by Somerman et al. (1988).[16] Briefly, biopsies of healthy gingiva (interdental papilla) were obtained from pre-molar of individuals undergoing tooth extractions for orthodontic reasons. The gingival samples was placed in a 15mL centrifuge tube (BD - Labware) containing "biopsy" medium - DMEM (Dulbecco's Modified Eagle Medium) with 10% fetal bovine serum (FBS), 250 μg/mL gentamicin sulfate, 5μg/mL amphotericin B, 100 μg/mL streptomycin 100U/mL penicillin (Gibco BRL, Life Technologies, Rockville, MD, USA). Next, gingival fragment was rinsed five times in the biopsy medium and cut in small pieces on a sterile Petri dish (35x100mm) with sterile scalpel. The microfragments were again placed in new Petri dish, arranged separately and then covered with DMEM supplemented with 10% FBS, 100 μg/ml streptomycin and 100U/mL penicillin G and incubated at 37 ° C, 5% CO2-95% air. After being characterized, oral fibroblast cells were used at passage 4 to realize this study.The medium was changed every 48 h until the cells surrounding the tissue explants were confluent. After this, the microfragments were removed, the cells trypsinized and plated into new wells for the experiment.

2.2. Identification of Candida

Sixteen strains of *Candida* ssp. were previously isolated from patients with chronic periodontitis and controlled insulin type 2 diabetes melittus. These strains were identified by PCR (Polymerase Chain Reaction) as *C. albicans* .The primers were designed from the gene AAT1a (ID 3643468) using the following pair of primers F: 3´- ACTGCTCAAACC

ATCTCTGG - 5´ and R: 3´- CACAAGGCAAATGAAGGAAT – 5´, with the fragment size of 452 pb.

2.3. Cellular Superficial Hydrophobicity Assay (CSH)

C. albicans strains were inoculated separately in 15 mL of SD broth (Sabouraud Dextrose, Difco Laboratories Detroit, MI) and incubated overnight at 37°C in 5% CO_2 atmosphere. Hydrophobicity assays were conducted according to Rodrigues et al., 1999. Briefly, yeast pellet were harvested and washed twice in phosphate buffer, pH 8.0. A cell suspension was prepared in the same buffer, obtaining an optical density (A0) of 0,4 – 0,6 (at 600 nm). Then, 150 µL of hexadecane was added to tubes containing 3 mL of this suspension, incubated at 30°C and mixed every 10 min, twice for 30s. After 30 min, phase separation was observed and optical density of the lower aqueous phase (A1) was measured and compared to that obtained prior to the mixing procedure (A0). The percentage of cells in the hexadecane layer (adhered cells) was used to estimate the hydrophobicity (H) using following formula: % H= A0 – A1/ A0 x 100%. This assay was performed in duplicate. We selected 16 strains previously isolated and identified in 8 HH (High Hydrophobicity) and 8 LH (Low Hydrophobicity).

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2.4. Adhesion and Invasion Assays

These tests were carried out according to the methods previously described by Cogo et al., (2009) [18] with modifications. Firstly, adhesion and invasion of *C. albicans* were assessed in fibroblasts obtained from primary culture of human gingival fibroblasts – GF (see details in the topic *gingival fibroblasts isolation***)**. The GF cells were maintained in RPMI medium supplemented with 10% fetal bovine serum (Cultilab,Campinas,SP,Brazil), supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 0.292 mg/mL L-glutamin), MEM, essential amino acids solutions (Gibco Laboratories) and 2.20 g/L sodium bicarbonate. For the experiments, GF cells were cultured in 25 cm^2 tissue culture flasks (Corning Inc., Corning, NY, USA) at 37°C in an atmosphere of 5% CO_2. From these culture flasks, around $1x10^5$ cells/well were inoculated into 24-well tissue culture plates

containing supplemented RPMI and incubated at 37 ° C in 5% CO2 for 24 h to reach a confluent monolayer of cells. Before the experiments, *Candida* spp. strains were grown in SD broth for 18 h and then were centrifuged at 4000g for 10 min to obtain the pellets for subsequent count of cells in hematocytometer. GF cells previously cultured for 24h were washed three times with phosphate buffered solution (PBS; pH 7.5) and a total of $1x10^3$yeasts/cm^2 was applied to these plates, obtaining an infection of 1:100 (GF:yeasts).

For adhesion assays, *C. albicans* were maintained in contact with fibroblasts for 2h and after this, wells were washed three times with PBS to remove unattached yeasts and the GF cells detached using 1 mL trypsin-EDTA (Gibco) diluted in PBS at 37°C for 2 min. An aliquot of 20µl was removed, serially diluted, plated on SDA and incubated for 48 h. The number of adhered yeasts in fibroblasts was assessed by counting the colony-forming unities/mL (CFU/mL). In the control group of adhesion test, culture was not washed with PBS and all unattached and attached yeasts were collected to obtain colonies counting and determination of CFU/mL.

For invasion assays, the experiment was repeated and after 4 hours of contact fibroblasts-yeasts in the same conditions, unattached yeasts were removed by washing three times the wells with PBS and killed by the incubation in RPMI containing 0.2µg/mL nistatin (SIGMA) for 30 min. After this, wells were washed three times with PBS and GF cells were disrupted by means of incubation in 1 mL of cold sterile water for 20 min. Cells lysates were serially diluted and plated on SDA plates that were incubated at 37°C for 2 days. Invasive yeasts were analyzed for determination of cfu/mL. For the control group in the invasion tests, cells were not submitted to washing with PBS and incubation with nistatin. Therefore all unattached and attached yeasts were collected to obtain colonies counting and determination of CFU/mL. All assays were performed in duplicate, in two independent experiments. For adhesion and invasion, the percentage was calculated based in each control group. For example: adhered cells x 100/control adhesion.

2.5. Measurement of Nitrite Concentration (NO)

The concentration of NO produced by gingival fibroblasts after 2 (adhesion) and 4 h (invasion) of incubation with *Candida albicans* were measured by the quantification of

nitrite, stable conversion product of NO. A standard procedure using Griess reagent (1:1 mixture v/v) of 1% sulfanilamide and 0,1% naphthylethylenediamine dihydrochoride in 5% H3PO4 was employed. Medium (100 µL) and Griess reagent (100 µL) were mixed and left 30 min. at room temperature. The samples were read at 550nm. [19] This assay was performed in duplicate.

2.6. Statistical analyses

Data concerning adhered and invasive *C. albicans* were analyzed by using ANOVA and statistical differences among the groups, according to hydrophobicity, were determined by Tukey´s post hoc. All statistical analysis was conducted at a significance level of 5% (SPSS, version 17.0).

3. Results

3.1. Cellular Superficial Hydrophobicity Assay (CSH)

Among the sixteen *C. albicans* strains, eight were considered highly hydrophobic (HH) and eight had low hydrophobicity (LH). The mean of percentage for hydrophobicity was statistically different between the groups HH (68%) and LH (3%) (Table 1). The CHS test ranged from 0.36% to 6.20% for the LH and 62.40% to 74.93% for the HH strains (Figure 1).

3.2. Adhesion and Invasion Assays

All strains were able to adhere and invade gingival fibroblasts but the strains that were considered as highly hydrophobic (HH) had statistically greater capacity to adhere and invade cells (Table 1). The mean of adhesion (in percentage) was 53.91% and 22.55% and for invasion were 24.4% and 9.8%, for HH and LH, respectively. The number of adhered cells was always higher than those invaded in both situations (HH and LH). (Figure 2). There was no difference between the HH and LH strains in the adhesion and invasion assays for the control groups ($p>0.05$). However, when controls were compared to adhesion and invasion groups, this difference was remarkable ($p<0.05$).

3.3. Measurement of Nitrite Concentration (NO)

NO production by fibroblasts was observed for all strains and there was an increase in the concentration with the time of exposure. The concentration of NO was higher in highly hydrophobic strains; however there was no statistical difference between HH and LH groups (Figure 3). NO production by fibroblasts was very high especially in contact with strains 2HH and 4HH both adhesion and invasion assays (Figure 4).

4. Discussion

Microbial adherence is an essential initial step in the infectious process. Besides the presence of microbial adhesins and receptors in host cells, microbial surface hydrophobicity has been described as an important factor that influences adhesion of microorganisms to biological or inert surfaces.[17] Cell surface hydrophobicity (CSH) plays an important role in mediating the adhesion of yeasts to epithelial cells, as well as to splenic, kidney, fibroblast and lymph node cells.[20] The increase of CSH also enhances the virulence of *C. albicans* in an animal model.[21] Experiments have suggested that high CSH impairs the phagocytosis, increasing resistance to blood clearance and consequently the virulence of *C. albicans*.[17] To understanding the pathogenesis of candidosis, *in vivo* and *in vitro* studies have been developed to characterize and quantify the adhesion of *C. albicans* in cell surfaces. [22,23] The binding of *C. albicans* to mucosal surfaces has been demonstrated as an important step in the infectious process particularly in the oral cavity. [22] The ability of *Candida* spp. to adhere to different cells is a determinant factor for dissemination, infection and persistence of these species in oral tissues. Nikawa et al (2006)[3] quantitatively evaluated the adhesion of oral strains of *C. albicans*, *C. tropicalis*, and *C. glabrata* to human gingival epithelial cells, gingival fibroblasts and pulmonary fibroblasts. They observed that the most of tested *Candida* strains had significantly more adherence to gingival epithelial cells than other types of fibroblasts. However, fibroblast cultures, primary or lineages cells have demonstrated to be a specific and reproducibly model for adherence and invasion of yeasts.[14] In the present study, both processes were successful assessed in fibroblasts. When compared to control groups, there was a significant reduction in *C. albicans* counting,

showing that after 2 or 4 h, adhesion and invasion may be observed for this specie in contact with human gingival fibroblasts.

Environment factors such as diet, composition of body fluids and presence of antifungal agents may also cause changes in the cell surface and thereby modulate *Candida albicans* adhesion.[24] Although many studies have demonstrated the adhesion process is related to bindings between host adhesins and mannoproteins of *C. albicans*, cell surface hydrophobicity (CSH) seems to have its importance in this process too. [22] Epithelial cells and fibroblasts are the primary components of the oral mucosa and any injury to these cells may lead to development of oral diseases such as candidiasis or periodontal disease.[25]Our results show that CSH is important factor non-specific in adhesion and invasion of *C. albicans* in oral fibroblasts. Highly hydrophobic cells were significantly more invasive than cells considered with low hydrophobicity in this study. Changes in the environmental conditions can trigger germination. Thewes et al., (2008) [1] assessed *"in vitro*" the invasion of *C. albicans* in human tissue cells (fibroblasts and epithelial cells) and found that the reduced formation of hyphae (a characteristic of the less hydrophobic cells) decreases the invasive properties and reduces the ability to invade tissue cells. The gingival pocket and gingival crevicular fluid are favorable environments for germination and hyphal growth of *C. albicans*. *Candida* hyphae have more ability to adhere to host surfaces and penetrate tissues compared with yeast cells.[9] This way, *C. albicans* could have a role in the infrastructure of periodontal microbial plaque and participate to adherence to the periodontal tissue.[9] In previous study conducted by present authors, cell surface hydrophobicity (CSH) in reduced oxygen and anaerobiosis were studied (data unpublished) and the results showed that strains were more hydrophobic when grown in anaerobic conditions. In anaerobiosis, *Candida albicans* appears in form of hyphae which is much more common than in other atmospheric conditions. Hydrophobic interactions may be of importance in promoting tissue invasion by filamentous yeast cells.[26]

Another virulence factor of *Candida* ssp. is their tolerance to innate and cell-mediated immunity that may be help the progression of inflammation in the surrounding tissues.[9] In the microenvironment of periodontal disease, besides the presence of several cytokines, has been observed that NO production acts directly on the maintenance or

progression of inflammation and tissue destruction.[15] When NO is locally produced in high concentrations has a cytotoxic or cytostatic effect against cells infected by fungi, bacteria, protozoa and tumor cells and adjacent cells resulting in extensive tissue destruction.[15] We investigated the NO production by gingival fibroblasts in the presence of *C albicans*. Our results demonstrated that gingival fibroblast was able to produce NO in the presence of *Candida albicans* after 2 and 4 hours of exposure. Interestingly, after 4 hours, NO production was detected in greater quantity than in two hours, suggesting that the longer the interaction between cells and the pathogen, the higher is NO production by cells. Highly hydrophobic strains have a tendency to stimulate an increase in the production of NO, although statistical difference was not found when these strains were compared to those less hydrophobics. The presence of this molecule in periodontal disease may reflect the involvement of an additional mediator in the regulation of bone resorption related to the progression of the disease.[27] The role of *C. albicans* in periodontal disease is not clear and further studies are needed to demonstrate the clinical significance of the findings. Only a few species of microorganisms normally found in diseased gingival pockets can penetrate the epithelia surface and provoke inflammation. In this study, we demonstrated the ability of *C. albicans* to adhere and invade human gingival fibroblasts, besides stimulate the production of NO by these host cells.

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Table 1. Mean (standard deviation) of values obtained for hydrophobicity of *Candida albicans* strains, adhesion and invasion of these strains in fibroblasts.

	Hydrophobicity %*	**Adhesion %**	**Invasion %**
HH**	68.89 A*** (4.66)	53. 91 A (9.13)	24.47 A (7.73)
LH	3.00 B (2.45)	22.55 B (8.27)	9.80 B (4.87)

* % - percentage; AN – absolute number of yeasts recovered in the process.
For adhesion and invasion, the percentage was calculated based in each control group. For example: adhered cells x 100/ control. See methods.
** HH – High Hydrophobicity; LH – Low Hydrophobicity
** For each parameter, means (standard deviation) followed by:
- Same uppercase letters in the rows are not statistically different, according to ANOVA and Tukey test ($p>0.05$).
- Same lowercase letters in columns are not statistically different, according to ANOVA and Tukey test ($p>0.05$).

Figure 1. Percentage of hydrophobicity by *C. albicans* strains isolated from diabetic and periodontal patients.

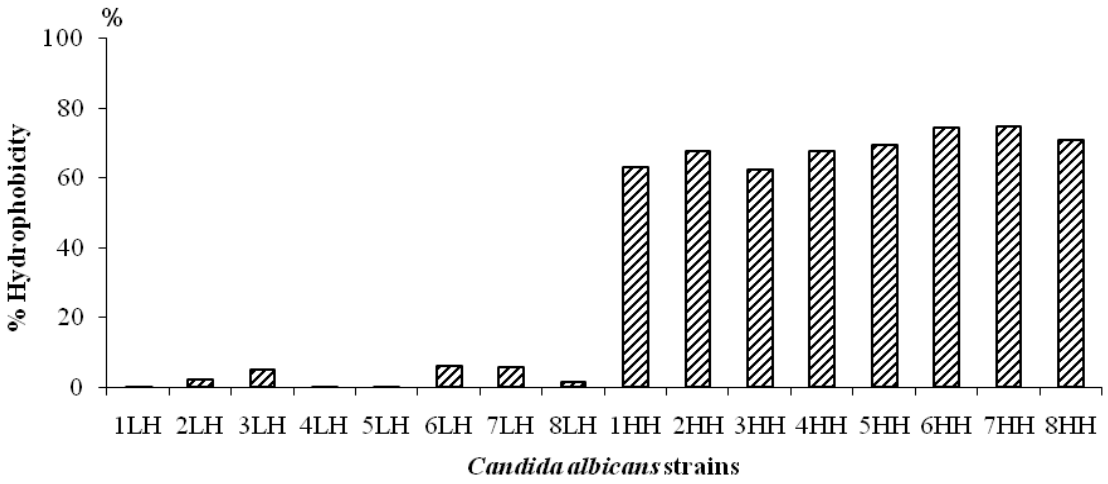
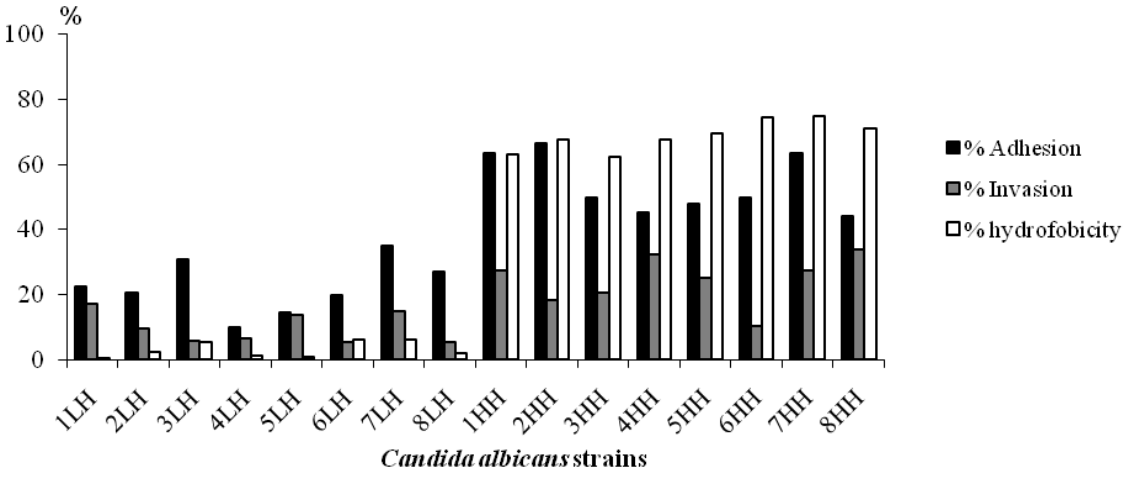
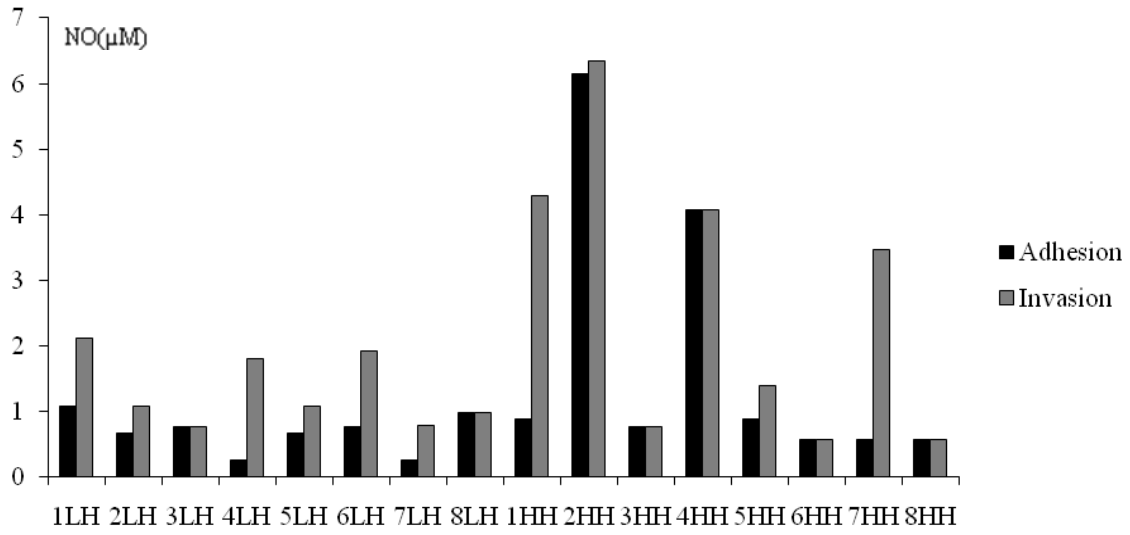


Figure 2. Percentage of adhesion, invasion and hydrophobicity of *C. albicans* strains in contact with human gingival fibroblasts.



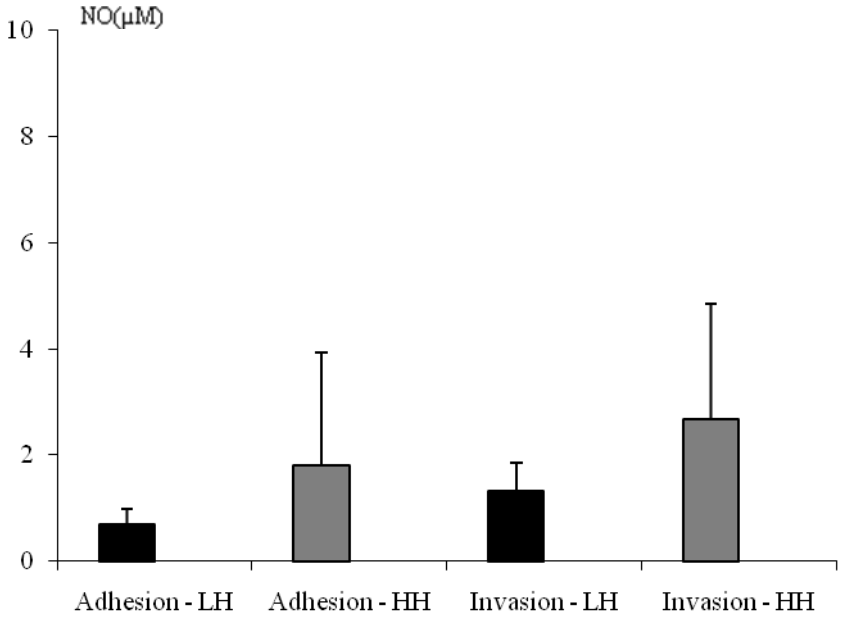
HH – High Hydrophobicity; LH – Low Hydrophobicity

Figure 3. Percentage of NO produced by *C. albicans* strains in adhesion and invasion assays.



HH – High Hydrophobicity; LH – Low Hydrophobicity

Figure 4. Means and standard deviation (SD) of NO concentration (µM) produced by fibroblasts in contact with *C. albicans* strains.



HH – High Hydrophobicity; LH – Low

7. CONSIDERAÇÕES GERAIS

Leveduras do gênero *Candida* são patógenos encontrados comumente em populações humanas, habitando as mucosas de diferentes sítios anatômicos, sendo capazes de residirem em equilíbrio com a microbiota do hospedeiro assintomático e imunocompetente. Contudo, podem comportar-se como patógenos oportunistas, produzindo doenças que vão desde lesões mucosas superficiais até disseminações sistêmicas graves e invasivas (Hannula *et al*.,2000). A cavidade bucal oferece uma variedade de nichos ecológicos à colonização microbiana, permitindo a sobrevivência de uma diversidade de bactérias, vírus e fungos (Socransky & Haffajee, 1994; Jabra-Rizk *et al*., 2001). *Candida albicans* têm sido detectada, em várias análises microbiológicas, infectando, inclusive, bolsas periodontais (Reynauld *et al*., 2001; Hannula *et al.,* 2001; Jawed *et al.,* 2009). Vários estudos relatam que as espécies de *Candida*, presentes na mucosa ou em áreas subgengivais, mesmo em indivíduos assintomáticos, podem contribuir tanto para a patogenese da doença periodontal como também aumentar os riscos de candidoses superficiais ou disseminadas, especialmente em caso de comprometimento imune (Nikawa *et al*.,1998; Jabra-Rizk *et al*., 2001; Starr, 2001; Barros *et al*., 2008).

Yuan *et al*. (2001) acreditam que bolsas periodontais que abrigam *C albicans* podem representar um importante reservatório desse fungo, favorecendo a ocorrência de candidose bucal. Para Hannula *et al*. (2001) é necessário discutir em que proporção as espécies de *Candida* participam da patogênese da periodontite, ou se são apenas comensais e inofensivas e ocorrem em bolsas periodontais apenas como resultado da difusão para o espaço subgengival em caso de colonização assintomática ou de candidose na mucosa bucal.

A transição do estado de comensalismo para o de patogenicidade é bem controlada nesses fungos, e atribuída à expressão seletiva de vários fatores de virulência, que agem de forma sinérgica, sob condições predisponentes favoráveis. Assim, o tipo, o estágio e o sítio de infecção, além da natureza da resposta imune, fazem com que a levedura expresse um ou mais de seus variados fatores de virulência (Hube & Naglik, 2001). Pelo fato de possuirem vários fatores de virulência relevantes na patogênese da doença periodontal, tais como a

capacidade de aderir ao epitélio e de invadir o tecido conjuntivo gengival (Järvensivu *et al.*, 2001), de inibir a função de neutrófilos polimorfonucleares (Maccarinelli *et al*, 2001), bem como produzir enzimas colagenase, proteinases e fosfolipases (Hägewald *et al*, 2002), as espécies de *Candida* poderiam agir como patógenos importantes em algumas formas de periodontite. Tem sido sugerido que os microrganismos podem adquirir vantagem seletiva na colonização das superfícies bucais se forem capazes de inibir a imunidade das mucosas pela degradação proteolítica da IgA. É provável que a proteólise dessa imunoglobulina facilite a penetração e disseminação de substâncias potencialmente tóxicas e ou de antígenos liberados pela microbiota subgengival. Esses processos podem desempenhar um papel importante na perpetuação das alterações inflamatórias associadas com as doenças periodontais (Hägewald *et al*, 2002).

Embora *Candida* spp. seja um microrganismo potencialmente patogênico de grande importância médica e odontológica, na doença periodontal ela tem sido muito pouco estudada. Dessa forma, este trabalho visou colaborar com informações sobre a presença de *Candida* spp. em diabéticos com doença periodontal, assim como elucidar o papel desses patógeno na progressão da doença. Em relação à análise por PCR de bolsas e furcas de pacientes diabéticos com doença periodontal, pudemos observar uma alta prevalência de *Candida* spp. no microambiente subgengival. Também notamos que as cepas isoladas de paciente diabético tipo II foram consideradas virulentas, em anaerobiose ou em concentração reduzida de oxigênio, e com capacidade de adesão e invasão em fibroblastos gengivais. No entanto, apesar das condições *in vitro* não representarem todo o universo de situações que ocorrem *in vivo*, esses achados auxiliam na compreensão dos diferentes mecanismos dessa espécie durante sua colonização na cavidade bucal. Finalmente, a presente tese, através dos resultados encontrados, pôde contribuir com o estudo da presença de *Candida* spp. em sítios periodontais e dos seus principais fatores de virulência, que poderiam auxiliar na progressão da doença periodontal, principamente em pacientes diabéticos.

8. CONCLUSÃO GERAL

Os resultados da presente tese conduziram às seguintes conclusões:

1. Espécies de *Candida* podem ser encontradas, em grande freqüência, até mais que bactérias denominadas periodontopatógenos, em bolsas periodontais e furcas de pacientes com periodontite crônica, portadores de diabetes mellitus tipo II.
2. A concentração de oxigênio no ambiente influencia os fatores de virulência das cepas de *C. albicans* isoladas de sítios subgengivais de pacientes diabéticos.
3. O genotipo A, obtido por PCR com primers especificos para regiões dos íntrons do gene 25S rDNA, parece ser mais virulento, considerando os testes fenotípicos (atividades enzimáticas e hidrofobicidade) avaliados neste estudo.
4. Cepas de *C. albicans* isoladas de pacientes diabéticos com periodontite crônica com alta hidrofobicidade apresentam maior capacidade de adesão e invasão em fibroblastos gengivais humanos e uma tendência a induzirem maior produção de óxido nítrico por essas células.

9. CONSIDERAÇÃO FINAL

A presença de *Candida* spp. e seu possível papel no desenvolvimento e/ou exacerbação da periodontite crônica em pacientes diabéticos ainda não foram esclarecidos, com base neste e em outros estudos científicos publicados, sugerindo que trabalhos futuros devam ser realizados.

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[1] De acordo com a norma da UNICAMP/FOP, baseada na norma da International Commitee of Medical Journal Editors – Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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11. ANEXO I

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Análise genética e dos fatores de virulência de cepas de Candida albicans isoladas de pacientes com periodontite crônica portadores ou não de diabetes mellitus**", protocolo nº **062/2008**, dos pesquisadores **JANAINA DE CASSIA ORLANDI SARDI, CRISTIANE DUQUE e REGINALDO BRUNO GONÇALVES**, satisfaz as exigências do Conselho Nacional de Saúde – Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 16/07/2008.

The Ethics Committee in Research of the School of Dentistry of Piracicaba - State University of Campinas, certify that the project "**Genetic analysis and virulence factors of Candida albicans strains isolated from diabetic or non-diabetic patients with chronic periodontitis**", register number **062/2008**, of **JANAINA DE CASSIA ORLANDI SARDI, CRISTIANE DUQUE and REGINALDO BRUNO GONÇALVES**, comply with the recommendations of the National Health Council – Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee at 16/07/2008.

Prof. Pablo Agustin Vargas
Secretário
CEP/FOP/UNICAMP

Prof. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.